ETHNIC VARIATION IN BREAST CANCER IN SINGAPORE

LI YUQING

NATIONAL UNIVERSITY OF SINGAPORE
2003

ETHNIC VARIATION IN BREAST CANCER IN SINGAPORE

LI YUQING
(B.MED, XI'AN MEDICAL UNIVERSITY, P.R. CHINA)

A THESIS SUBMITTED

FOR THE DEGREE OF MASTER OF CLINICAL SCIENCE

DEPARTMENT OF MEDICINE

NATIONAL UNIVERSITY OF SINGAPORE

2003

Acknowledgements

I am very grateful to the Department of Medicine, National University of Singapore for providing a very inspirational, helpful, supportive environment and scholarship for making the conception, implementation, and documentation of this thesis a possibility. I would like to dedicate my gratitude to Prof. Lee K.O for his invaluable advice, numerous helpful ideas, encouragement and guidance ranging from research work to life philosophy. I am very grateful to Senior Scientist Peter E. Lobie for his suggestions, friendship, and wisdom.

I am also very grateful to Associate Prof Adeline Seow for being an outstanding teacher with enthusiastic advice when the task ahead of me appeared too monumental for my chisel. Much gratitude goes to Dr Lim Siew Eng, for being a continuous supportive friend and source of solving samples problems. Thanks to the whole Endocrine and Signal Transduction lab in IMCB for late night company and support. Special thanks to Joyce, Fu Li, Ralph, and Zhu Tao for continuing support and rendering of help throughout the years.

Last but not least, I want to deeply thank my husband, Zhou Xiaowei, for accompanying me in the hardest time of my life, putting up with me during the time I spent on this thesis. I also want to sincerely thank every other person whom I may not have mentioned here, but who is always in my heart of hearts.

Table of Contents

Summary

The incidence of breast cancer has increased sharply among women in the last 3 decades in Singapore. The aim of the present thesis is to investigate various aspects of breast cancer in Singapore, using epidemiology and experimental studies. We investigated the epidemiology of ethnic variation (Chinese, Malay and Indian) in breast cancer in Singapore women. In addition, we carried out preliminary studies on leptin, which is related to body mass index, on a breast cancer cell line in vitro.

To study the epidemiology of ethnic variation in breast cancer, the number of cases and incidence rates of breast cancer from 1968 to 1998 were obtained from the Singapore Cancer Registry Report. In addition, a case-control study was designed and the results were analysed by logistic regression. Bivariate odds ratios (OR) for risk of breast cancer and 95% confidence intervals (95% CI) were calculated for ethnic status. Our study showed that although the three main ethnic groups have had striking increases in incidence of breast cancer over the past three decades, the pattern of increase was different. The greatest incidence rate was in the Chinese group while the highest annual increase was in the Malay group. The Indian group had the highest post-menopausal breast cancer incidence while Chinese group had the highest incidence in pre-menopausal breast cancer. The case-control study showed that while Chinese, Malay and Indian women shared some common risk factors for breast cancer, other factors related to breast cancer were distinguishable between the three ethnic groups. Cycle length of menses period, menopause status, age at first marriage, number of full term pregnancies, age at first pregnancy and oral contraceptive use were in this subset of distinguishable factors

related to breast cancer risk among Chinese, Malay and Indian women in Singapore. These factors may help to provide an explanation for different ethnic patterns for incidence rates of breast cancer.

In the experimental studies (Part II of this thesis), the results demonstrated that the role of leptin on mammary carcinoma cell line (MCF-7) proliferation was mediated by the specific leptin receptor in vitro. We found that the leptin receptor is expressed on the MCF-7 breast cancer cell line and it is activated by leptin via the Mitogen-activated protein kinase (MAP-kinase) pathway. Reverse transcription-polymerase chain reaction (RT-PCR) study demonstrated the existence of leptin receptor mRNA in MCF-7 cell line. Double labeling cofocal Laser scanning microscopy also confirmed the existence of leptin receptor in the same cell line. Western blot demonstrated the leptin receptor protein expression in MCF-7 cells. Further, P44/42 MAP Kinase activity was increased by 100ng/ml human recombinant leptin in a dose and time dependent manner. Cell proliferation, assessed with 5-bromo-2-deoxyuridine (BrdU) uptake into MCF-7 cells, was also significantly increased by the incubation of 100ng/ml leptin for 24hours, compared with samples which had no leptin, or had addition of specific inhibitor of P44/42 MAP Kinase, Mek ½ inhibitor (U0126). Finally, cDNA microarray was used to investigate other leptin effects on the MCF-7 breast cancer cell line. In conclusion, the leptin receptor exists in the MCF-7 human breast cancer cell and through its specific leptin receptor, leptin has the ability to stimulate breast cancer cell proliferation in vitro.

List of Tables

List of Figures

List of Publication

Ethnic differences in trends in breast cancer incidence in Singapore (Abstract)

Y.Q. Li, A Seow, P E Lobie, S E Lim, K O Lee

International Journal of Cancer Supplement 13:P228. (18th UICC International Cancer Congress)

Chapter 1 Introduction

1.1 Breast cancer in Singapore

The incidence of breast cancer in Asian countries has increased sharply over the past three decades. In Singapore, the number of women diagnosed with breast cancer has increased at a mean rate of 3.5 % annually since 1968. Breast carcinoma is currently the most common female cancer, accounting for 3574 new cases during 1993-1997. Up to 20% of all cancers diagnosed in Singapore women is breast cancer. The age-standardized rate doubled from 20 (1970) to 40 (1990) (per 100,000 per year) and has been extrapolated to reach 55 (per 100,000 per year) by the year 2000.

In the last 30 years, Singapore has transited from a developing to a newly industrialized economy country and accompanying this change, Singaporeans also have experienced great changes in lifestyle, environment, and patterns of disease. The rise in breast cancer incidence, and the possibility of ethnic variation in this rise, has not been studied previously. Although genetic causes play an important role in breast cancer, the rapidity of the change in incidence suggests that environment changes, lifestyle changes, may be the important or even dominant factors.

1.2 Ethnic variation of breast cancer in Singapore

The incidence of female breast cancer varies markedly between countries and ethnic groups. The highest incidence rates are in the United States and Northern Europe,

intermediate in Southern and Eastern Europe, and lower in Asia and the Far East. In Singapore, which comprises of three main ethnic groups, there may be differences in ethnic incidence rates for breast cancer.

Different lifestyles exist in the three main ethnic groups in Singapore women. It is now widely accepted that life-style related factors such as, reproductive and body mass index are associated with risk of breast cancer. These factors maybe partially explain the ethnic variation in the risk of breast cancer.

1.3 Aims of the present study

Singapore is a multi-ethnic country. In the 1990 census the total population was 3,016,379, comprising 77.7% Chinese, 14.1% Malays, 7.1% Indians and 1.1% others [Lau, 1992].

We noted a trend in the breast cancer incidence among Singapore women from 1968 to 1997 had uneven increase among the different major ethnic groups. In this thesis, the aim will be to update the analysis of the overall breast cancer trend in Singapore following a primary study "Trends in incidence 1968-1992" [Seow A et al, 1996]) and in particular, describe the differences of incidence rates of breast cancer among the three ethnic groups.

We also aimed to determine the risk factors for breast cancer in Singapore women by carrying out a pilot case-control study. We hypothesize that women with breast cancer would have a higher proportion of identified risk factors compared with the control

women. We aimed to compare the established risk factors of breast cancer among different ethnic groups in our subjects. Specifically, we aimed to examine the role of some hormone related risk factors in variation incidence rates among different ethnic groups.

Finally, in this thesis, we were also interested to examine the role of leptin, a hormone that is closely associated with the body mass index, to investigate its involvement in the metabolic pathways of human breast caner cell. This is described in Part II of this thesis.

Chapter 2 Literature Review

The incidence of breast cancer has been steadily rising since formal registration of this tumor began in the 1930s. From 1940-1982, the age-standardized incidence rate has risen by an average of 1.2% per year in Connecticut of US, the state with the longest continuous cancer registration [Miller, et al, 1991]. In both industrialized and developing countries, similar long-term increases are being observed [Boyle, et al., 1990; Prentice, et al. 1990].

In Singapore, the breast cancer incidence rate rose from 134 per 100,000 in 1970 to 715 per 100,000 in 1995 [Chia, et al, 1996& 2000]. This increase has occurred in both younger and elder women. Some of the recent increase in breast cancer incidence rather than mortality is likely to be partly the result of diagnosis of breast cancer at progressively earlier stages and, hence, higher 5-year survival rates. But the most important reason of sharply increasing rate may be still due to the risk factors of breast cancer induced by factors in the modern lifestyle.

Risk factors for breast cancer have been extensively investigated. Most of the population-based studies have been done in Europe and other Western countries, which suggest that hormonal-related factors variation induced by modern lifestyle, such as reproductive factors, body mass index, predispose specific populations to higher risk.

In this section, risk factors of breast cancer, hypotheses for the relationship of hormonal related factors and variation of breast cancer incidence rates are reviewed. In addition, as Singapore is a multi-ethnic country, and as this thesis will investigate ethnic variation in breast cancer, previous research related to hormonal risk factors and ethnicity will also be reviewed.

2.1 Risk factors of breast cancer

Despite the large number of risk factors, few are strongly associated with the development of breast cancer, and no single factor or combination of factors can predict the occurrence of breast cancer in any one individual. Through numerous epidemiological studies, an array of breast cancer risk factors has been established (Table2.1.1).

The risks associated with reproductive variables of never having children, being of a late age at first birth, having an early menarche, having a late menopause, are thought to be related to the hormonal environment to which the breast is exposed (during pregnancy or during a long menstrual history). The high body mass index is thought to be helpful to decrease the risk of breast cancer in premenopausal women, whereas it is a risk factor for postmenopausal women [Ng, et al, 1997; Le, et al 1988].

Several other breast cancer risk factors have been examined, but the results across studies are inconsistent. These include use of exogenous hormones, notably oral contraceptives and estrogens replacements during menopause.

Although there are only a few well-designed studies, most that are published have shown decreased risks of breast cancer among women who are more physically active with regular exercise. There is also limited evidence of an increased breast cancer risk among women who smoke or have the genetic predisposition to be slow acetylators of aromatic amines. Passive exposure to environmental tobacco smoke may also be a risk factor of breast cancer [Adlercreutz et al, 1990].

Table 2.1.1 Factors that influence breast cancer risk

Established Positive Risk Factor	Established Negative Risk Factor
1. Increasing age 2. Early menarche 3. Late menopause 4. Proliferative breast disease 5. Family history of breast cancer 6. Postmenopausal obesity 7. Late first term pregnancy or nulliparity 8. BRCA1 or BRCA2 mutations	1.Early bilateral oophorectomy 2.Premenopausal obesity
Possible Positive Risk Factors	**Possible Negative Risk Factors**
1. Postmenopausal estrogen replacement therapy 2. Oral contraceptive, long-term use at young age	1. Breast feeding 2. Physical activity (exercise) 3. Irregular menses, with long interval between menses

Source: Darcy et al. 1998

2.1.1 Endogenous Hormonal Factors

2.1.1.1 Endogenous Estrogen

Estrogens stimulate division of the breast epithelial cells, and increased cell division increases the chance of mutation occurring. It therefore has been hypothesized that breast cancer risk increases with increasing concentrations of estradiol in the serum. Several comparisons of estrogen levels between low and high risk ethnic groups [Dickinson et al, 1974; Glodin et al, 1986; Key et al, 1990; Shimizu et al, 1990], but not all [Trichopoulos et al 1984, Goodman et al, 1988], support the idea that populations with low breast cancer risk have lower levels of endogenous estrogens than women in high risk populations.

In addition to levels of endogenous estrogens, variations in estrogen metabolism have been investigated [Kabat GC et al; 1997, Ursin G. et al, 1999]. Estradiol is metabolized in two main competing pathways, via 16α-hydroxylation and 2-hydoxylation, and probably also via a minor 4-hydroxylation [Nebert DW, 1993; Yager JD et al. 1996]. It has been proposed [Yager JD et al. 1996; Bradlow HL, et al, 1995] that women who metabolize a larger proportion of their endogenous estrogen via 16α -hydroxylation are at greater risk. The reason is that 16α-hydroxylation has genotoxic effects, damages DNA, and enhances breast cell growth, whereas 2-hydroxyestrone inhibits breast cell proliferation although 2-hydroxy compounds also appear to have some estrogenic and growth promoting effects [Yager JD et al. 1996]. A study from Singapore [Ho GH, et al, 1998] also detected a highly protective effect of the 2-hydroxyestrone to 16α -hydroxyestrone ratio.

2.1.1.2 Endogenous hormone related factors

Although studies on endogenous estrogens are still inconclusive, the circumstantial evidence that estrogens contribute to breast cancer risk is strong.

Numerous studies have shown significant association of breast cancer with the age at menarche, menopause, and first pregnancy. Based on the statistics from NIH (National Cancer Institute of USA) cancer registry [Ries et al. 1994], the absolute age-specific incidence of breast cancer is higher in postmenopausal than premenopausal women, while the rate of the rise of the curve reaches the highest point to the time of menopause, then slows down to one-sixth in the premenopausal period. This has led to the suggestion that ovarian activity plays a major part in the causation of breast cancer [Henderson et al. 1988]. An increased number of ovulatory cycles has been suggested to be the common mechanism of increased risk.

Several researchers [Madigan, et al 1995; Rockhill, et al 1998; Bruzzi, et al 1985; Seidman, et al 1982] have developed models to estimate the attributable breast cancer risk due to menstrual and reproductive risk factors, resulting in estimates between 21% and 55%. A comparison between breast cancer risk factors in China and in the United States [Smith-Warner, et al 1998] found that the combined population attributable risk was 44% for reproductive factors, 32% for anthropometric factors, and 63% for all factors combined.

Between 1994 and 1996, a prospective case-control study [Ng, et al, 1997] was conducted among Chinese women in Singapore to investigate for factors associated with the risk of

breast carcinoma ages 45 to 69 years. Reproductive and menstrual factors significantly related to risk for breast carcinoma were number of deliveries (OR=0.81; 95%CI, 0.7-0.9; P<0.001), age at last delivery (P=0.03), and use of hormone replacement therapy (OR: 0.54; 95% C.I, 0.3~0.9; P<0.01).

Known breast cancer risk factors [Henderson, et al 1982] can be understood as measures of the cumulative exposure of the breast to estrogen and, perhaps progesterone, as a result of the frequency of ovulatory menstrual cycles. Therefore, both a late menarche and an early menopause decrease risk, probably by reducing lifetime exposure to these ovarian hormones.

Studies of the effect of lactation on breast cancer risk have also been inconclusive [Layde PM, et al, 1989; Kvale G and Heuch I, 1988]; but recent studies have suggested that a long duration of lactation reduces breast cancer risk in premenopausal women [Newcomb PA, et al, 1994].

In 1981, Pike et al published their notable work showing that young women (under age 32) who had experienced an abortion before their first live birth had a 140% increased risk of breast cancer. A number of studies followed but finally in 1994, Daling et al published a large study, which noted that women who had an abortion before first birth suffered a 40% increased risk, and that this increased to 150% if the abortion was before age 18. Finally, in 1996, in what is openly regarded as the most meticulously comprehensive meta-analysis of all the abortion/breast cancer research studies ever done, Brind et al, [1996] found that women who had an abortion before their first term child had a 50%

increased of developing breast cancer while women who had an abortion after their first child sustained a 30% increased risk. This has not been completely accepted, and some studies [Davidson, 2001; Sanderson, et al. 2001] do not show any significant association.

As we all know, when a woman becomes pregnant, a number of hormone levels increase dramatically in her body. Three especially notable ones are estradiol, progesterone (i.e., the female sexual hormones), and B-hCG (Beta-human Chorionic Gonadotropin). All of these hormones, especially the last, serve to stimulate immature breast cells to mature into fully differentiated cells [Russo J and Russo IH, 1994]. If this process gets artificially interrupted, by way of an induced abortion, the hormone levels drop dramatically thereby suspending the natural process of maturation of many of the woman's breast cells. This is referred to as a "hormonal blow" by researchers. These cells are now "vulnerable" to carcinogens since they were left "in limbo": that is, they started the maturation process, but were never able to complete it. Cells that have fully matured are less vulnerable to mutagenic carcinogens than cells that are in the process of maturation.

2.1.2 Exogenous Hormonal Factors

Oral contraceptive (OC) use and its possible relation to breast carcinoma risk have generated debate for the last several decades. The literature was reviewed in detail by Spicer and Pike, with women stratified by menopausal status at the time of diagnosis of breast carcinoma [Spicer, et al 1994]. They studied 5 population-based case-control studies and 3 cohort studies of women younger than 45 years old. All 8 studies demonstrated an increased risk of breast carcinoma in OC users. In contrast, the 3

population-based case-control studies and 2 cohort studies of women diagnosed with breast carcinoma after age 45 years showed no effect with OC use. The magnitude of the increase in risk for premenopausal women at diagnosis was estimated to 3.1% per year of OC use [Spicer, et al 1994].

Other studies have examined the use of OCs in African American populations. Mayberry demonstrated an increased risk with OC use for black women ages 20-39 years, but such an increase in risk with OC use was not observed for the group ages 40-54 years [Mayberry, et al 1994]. Similar effects of OC use were observed by Palmer et al [Palmer, et al 1995]. Among black women younger than 45 years, 3 or more years of OC use was associated with increased risk of 2.8 (95% CI, 1.5-2.0), with no association observed for women ages 45-59 year. Brinton et al (1997) observed a reversed pattern for the effect of OC use by age at diagnosis. In that study a stronger risk with the duration of OC use was observed for black women ages 40-54 years. Overall, the epidemiological studies seem to indicate an increase in risk of breast carcinoma with OC use in premenopausal but not postmenopausal white women, with less consistent results for black women. This ethnic variation within U.S.A has not been emphasized much and may be relevant to the findings in Singapore presented in this thesis.

OCs contain many of the same hormones as HRT, albeit in different doses and ratios. By analogy, then, since exogenous estrogen-progestin combinations may be associated with breast carcinoma in premenopausal women, the same may be true for postmenopausal women taking estrogen replacement therapy with or without progestin.

In addition, estrogen replacement therapy could not account for the difference in breast cancer risk between Japanese and Caucasian women in a study in Hawaii [Nomura, et al, 1986]. The possible importance of bio-available estradiol fractions determined by sex hormone-binding globulin, the proportion of free estradiol, and progesterone has been suggested as a factor for the differences [Henderson, et al 1982; Pike, et al 1993].

2.1.3 Anthropometrics

In studies of breast cancer, anthropometric measurements may serve as useful biologic markers of environmental factors, including contemporary and past diets. The importance of body weight and body mass index (BMI) as risk factors among women has been well documented [Eugenia E, et al, 2003; Ng, et al, 1997; Le, et al 1988].

A case-control study from Hawaii [Le, et al 1988] described a protective effect of adolescent body mass against premenopausal breast cancer and an association between adult weight and postmenopausal breast cancer risk. Among women with Asian ancestries in the United States [Zidgler, et al 1996], adiposity conferred twice the breast cancer risk and recent weight gain of more than ten pounds tripled the risk, a stronger effect than usually found among Caucasian women [Hunter, et al 1993]. Despite the low levels of BMI in Japan, BMI [Hu, et al 1997] and weight gain [Hirose, et al, 1999] were confirmed as predictors of postmenopausal breast cancer risk.

Another two studies of anthropometrics have been conducted in Asian countries. A breast carcinoma case-control study in Singapore Chinese women ages 45-69 years (n=204 cases and 882 controls) found that although obesity did not predict risk for breast carcinoma, central obesity was strongly and significantly related to risk [Ng , et al 1997]. Women in the highest quintile of waist-to-hip ratio were >9 times (95% confidence interval, 4.6-17.5) more likely to develop breast carcinoma compared with women in the lowest quintile (p=0.0001). In Japanese women in Japan, women who were in the highest quartile for body mass index had double (95% CI, 1.49-2.03) the risk for breast carcinoma compared with women in the lowest quintile [Hirose, et al 1999]. Furthermore, they found that weight gain in later life was associated positively with risk for breast carcinoma, regardless of body mass index in early adulthood.

These studies are consistent in indicating that postmenopausal obesity is associated with the increased risk [Dewaard F, Baanders-van Halewijin E, 1974]. This may be due to increased peripheral estrogen production in postmenopausal women who have more adipose tissue, and therefore it increases the breast cancer risk. But this relationship is not observed in premenopausal women.

Height was associated with breast cancer among women of Japanese ancestry but not among Caucasian women living in the United States [Kolonel, et al, 1986]. Whereas it has been proposed that height is related to breast cancer risk primarily in populations where inadequate caloric intake in childhood and adolescence limits growth, it appears that taller women in some populations with a sufficient food supply also experience a higher breast cancer risk [Hunter, et al, 1993]. Possibly, inherited patterns in endogenous hormones and

growth factors determine the height reached after puberty and at the same time contribute to the promotion of breast carcinogens.

Physical activity in adolescence is reported to decrease risk, perhaps due to a higher rate of anovulatory cycles [Frisch R, et al, 1981; Bernstein L, et al, 1994], but an increased level of physical activity later in life has not been shown to reduce breast cancer risk. [Dorgan JF, et al, 1994]

2.1.4 Diet

Based on ecologic evidence and on results from cell and mammal studies [Adlercreutz, et al 1990], the role of isoflavones contained in soy products [Adlercreutz, et al 1990; Messina, et al, 1991; Nomura, et al 1978; Wu et al, 2002] has been investigated in breast cancer prevention.

A case-control study among Asian-American women [Wu, et al 1996] detected a 30% decreased risk of breast cancer for women who reported eating tofu more than once a week as compared to women who ate tofu less than once a month. The estimated relative risk for breast cancer was 0.4 in a study from Singapore [Lee, et al 1991] when premenopausal women who consumed 55 g or more soy products per day were compared to women who consumed less than 12 g of soy products per day, but these results were not confirmed in a Chinese study in Shanghai and Tianjin [Yuan, et al 1995]. Although fruit and vegetable intake may protect against breast cancer [Freudenheim, et al 1996], the low

fruit and vegetable intake in Japan make it an unlikely candidate to explain the low breast cancer risk in this population.

2.1.5 Immigrant studies

Studies of migrants suggest that the environment is an important factor that is responsible for the variation in breast cancer rates among countries. However, the speed with which incidence rates among migrants and their offspring approach those of their adopted country has varied considerably from one study to another and from one ethnic group to another.

One study [Buell, et al, 1973], for instance, found that first-generation Japanese migrants to the United States had slightly higher breast cancer incidence rates than women in Japan, but incidence rates in second-generation migrants, who were potentially exposed to a new environment and culture at an early age, increased more markedly.

The pace of accumulation may differ among various ethnic groups. The slower rises in breast cancer rates among Japanese, Chinese, and Mexican migrants to the United States and their offspring as compared with Polish migrants suggested to another group of investigators [Thomas, et al, 1987] that either some protective factor in the former cultures is carried over into the second generation or that some risk factor for breast cancer is avoided by these second-generation individuals, as well as by their migrant parents.

A study in Los Angeles [Shimizu, et al, 1991] found that the age of migration of Asians and Hispanics affects risk: those who migrated at an early age had much higher incidence rates than those who migrated in adulthood, a finding which suggests that either some exposure early in life or total years since migration is of etiologic importance. These observations are interesting and suggestive, but the difficulties in studying them have prevented any further development.

2.1.6 Genetics

Recent studies have suggested that about 10% of breast cancer cases are directly due to inherited mutations in breast cancer related genes and that most of these result from mutations of the BRCA1 and BRCA2 genes. Normally, these genes help to prevent cancer by making proteins that keep abnormal cells from growing. However, if a person has inherited a mutated gene from either parent, chances of developing breast cancer increase. About 50% to 60% of women with inherited BRCA1 or BRCA2 mutations will develop breast cancer by the age of 70 [Inoue R, et al, 1995]. Women with these inherited mutations also have an increased risk for developing ovarian cancer. Inherited mutations of the p53 tumor suppressor gene can also increase a women's risk of developing breast cancer, as well as leukemia, brain tumors [Hartmann, et al, 1996]. Recently, Sng et al [Sng JH, et al, 2000] has reported data on BRCA1 mutation in Chinese patients will early onset breast cancer in Singapore.

With the development of molecular epidemiology [Perera, 1996], so-called biomarkers of susceptibility have been identified. Polymorphisms of genes coding for activating (phase

I) and detoxifying (phase II) enzymes may be associated with susceptibility to certain cancers [Le, et al, 1998].

In addition to carcinogens [Le, et al, 1998], several dietary agents, e.g., indole-3-carbinol [Michnovicz, et al, 1991], isothiocyanates [Shapiro, et al, 1998], and quercetin [Obermeier, et al, 1995], appear to be able to induce phase I or II enzymes, thereby leading to an interaction between genetic susceptibility and environmental exposure.

2.2 Hypothesis for the relationship of hormonal, reproductive related factors and breast cancer incidence rates variation

Several hypotheses have been submitted to explain the relation between hormonal, reproductive related factors and breast cancer incidence rates variation. One of the hypotheses noted that when the age-incidence curve of breast cancer is plotted on a log-log scale, the curve produced is not a straight line as it is with other cancers [Pike, et al. 1983]. Instead, the curve assumes a straight line until approximately age 50 years, when a decrease in the slope is noted. This observation has pointed towards the etiology of breast cancer and its dependence on female hormones for the induction and promotion of carcinogenesis.

2.2.1 Pike's "Breast Tissue Age" Model

As proposed by Pike's model [Pike, et al, 1983], the international variations in breast cancer incidence may be a result of differences in hormonal, primarily estrogen, exposure

leading to aging of the breast tissue according to breast tissue age, rather than according to chronologic age [Henderson, et al 1982, Henderson et al 1996].

The physiology of the female breast is dependent on the mammary proliferation effects of the ovarian hormones, estrogen and progesterone. Estrogen is primarily responsible for elongation and branching of the breast ducts, whereas progesterone is necessary for lobular development and maturation [Topper, et al. 1980]. The high levels of circulating hormones during pregnancy result in the differentiation of the terminal duct-lobular unit (TDLU), which is the major site of malignant transformation in the breast. This process of differentiation of the TDLU is protective against breast carcinoma development, and its effect is permanent [Russo, et al, 1995].

The theory of Pike assumes that breast carcinoma incidence rates vary in proportion to a power of the accumulated "breast tissue age" [Pike, et al, 1983]. In that model, the breast tissue ages at a constant rate between age at menarche and age at FFTP (First Full Term Pregnancy), at which time the hormonal milieu of pregnancy causes a one-time increase in breast tissue age but lower the rate of subsequent " breast tissue aging". This lower rate continues until the perimenopausal years, at which time it decreases linearly until menopause. After menopause the breast tissue continues to age, but at a much lower rate. This model predicts that at a given age, a woman with an FFTP (first full term pregnancy) in the preceding 5-10 years is at increased risk relative to a nulliparous woman. Studies of Janerich and Hoff, Lubin et al., and Pathak et al. have confirmed this theory [Janerich, et al, 1982; Lubin, et al, 1982; Pathak, et al, 1986]. Carrying a pregnancy to the third trimester confers a protective effect on lifetime risk for breast carcinoma if that pregnancy

occurs early in life. However, if a women delivers her first child close to the age at which menopause occurs, her lifetime risk is actually higher than if she was nulliparous.

2.2.2 Pathak and Whittemore's model

Pathak and Whittemore [Pathak, et al, 1992] extended Pike's single-birth model to a multiple-birth model, by incorporating a smaller increase in risk at each additional full term pregnancy with a subsequent lowering of the rate at which breast tissue ages. Rosner et al. [Rosner, et al, 1994] fitted the extended Pike model to prospective data from the Nurses' Health Study and obtained breast carcinoma incidence curves for various combinations of age at FFTP, total parity, and ages at subsequent births. Based on his model, the predicted breast carcinoma incidence rates started at age 30 years up to age 70 years for nulliparous women, women with 1 birth at age 20 years, women with 2 births at ages 20 and 23 years, and women with 3 births at ages 20, 23, and 26 years. The predicted incidence curves for these hypothetical scenarios show lower risk for nulliparous women relative to multiparous until ages 42-45 years, at which time a crossover in incidence occurs and the multiparous are at a lower risk relative to the nulliparous women. The crossover for women with a single birth at age 20 years does not occur until age 55 years. This shift to a later age for the crossover effect for women with a single birth relative to multiparous women would be expected based on the hypothesized decreased rate of breast tissue aging after each subsequent pregnancy.

2.2.3 Effect of hormones on carcinogenesis

Hormones induce carcinogenesis by inducing cell proliferation, which is an essential component of carcinogenesis [Pike, et al, 1993]. This hypothesis is explained by the observation that increased cell proliferation results in a larger pool of cells that are susceptible to defective DNA repair. This in turn leads to mutations, which are subsequently propagated through increased mitotic activity present in proliferating cells [Preston, et al, 1990], and can result in cancer formation. In contrast, differentiation of cells in the terminal duct-lobular unit (TDLU), produces the long term effect of slowing the cell cycle in the epithelial cells of this location, which allows more time for DNA repair, which in turn will lead to decreased carcinogenesis [Colditz, et al 1995].

2.3 Ethnic variation in breast cancer and its association with hormonal related risk factors

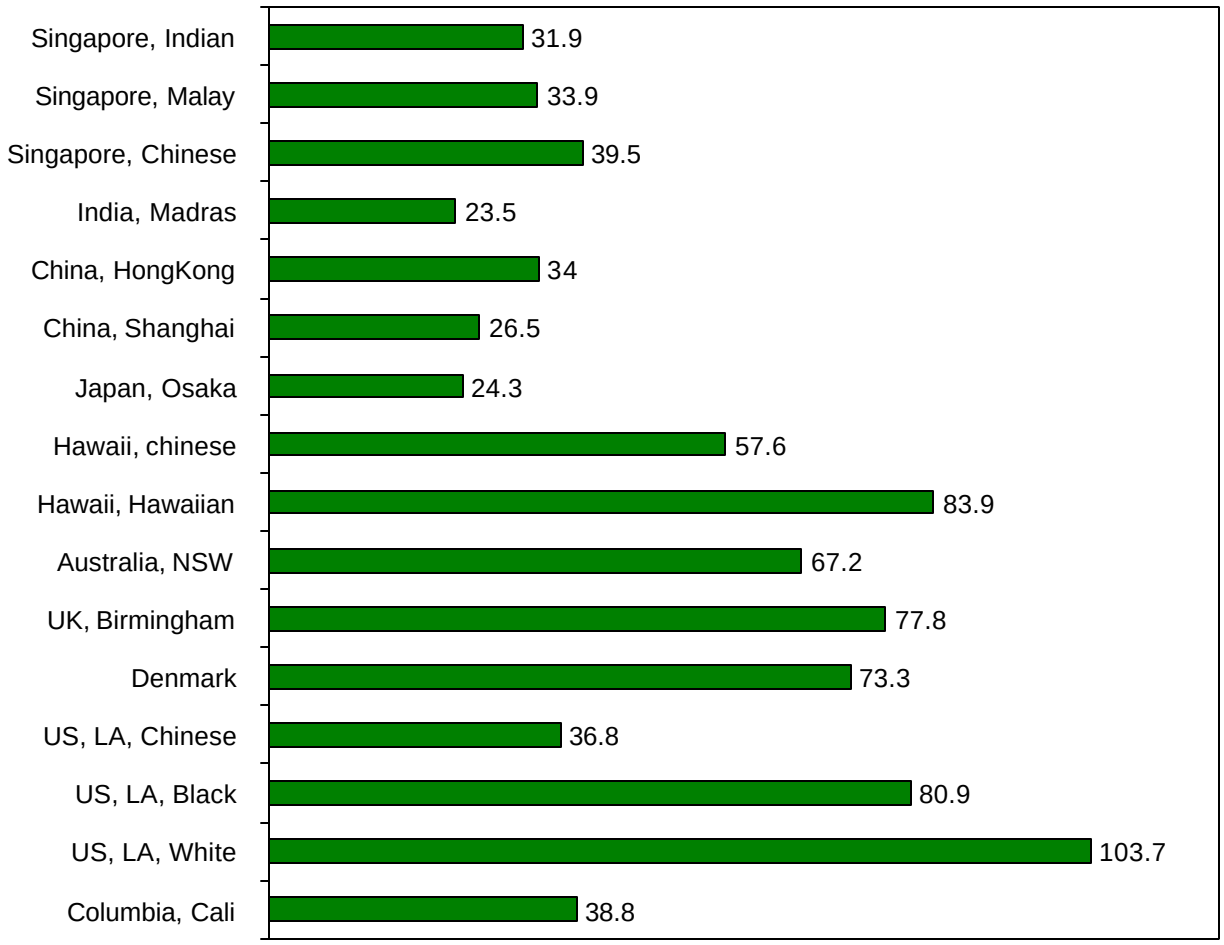
There are considerable differences in the incidence rates of breast cancer in different populations. The incidence rate in Singapore is about one-third of that of American women and half that of European women. It is estimated that while an American woman has a 12% chance of developing breast cancer in her lifetime, in Singapore the chance is lower, estimated at 4 to 5% [Parkin et al, 1992]. The high-risk group is composed predominately of white population. The low risk group is composed predominantly of Asian and African populations. The intermediate group is made up of westernized blacks and populations of Latin extraction. The Asians have low rates if they remain in their own country but acquire higher rates if they migrate geographically like the Japanese Hawaiians. This distribution is therefore telling us that the determinant is obviously not only geography but also diversity in the groups of women.

2.3.1 Ethnic variation of breast cancer incidence rate

Figure 2.3.1 illustrates the marked variation in the age standardized incidence rate of breast cancer among different countries between 1988 and 1992 [Chia, et al, 2000]. The incidence rate of breast cancer in Singapore was lower than UK (77.8 per 100,000), US and other western countries. However the women of all the three major ethnic groups (Chinese, Malay and Indian) in Singapore had higher age standardized breast incidence rates than other Asian countries. For example, the incidence rates in Singapore were higher than their corresponding ethnic group in Shanghai of China (26.5 per 100,000) and Madras of Indian (23.5 per 100,000).

Breast cancer incidence has been increasing in women all over the world. In the multiethnic study in Hawaii, over the last forty-years, significant increases in breast cancer risk have been observed in all ethnic groups except Chinese [Galanis, et al, 1998]. Although breast cancer risk in Hawaii among women of Japanese and Filipino ancestry remains lower than among Caucasian women, Japanese and Filipino women experienced a greater increase in risk than did Caucasian women. While the risk approximately doubled for Caucasian and Native Hawaiian women, it quadrupled for Japanese women and tripled for Filipino women. The limited data available for Chinese, who constitute less than 10% of Hawaii's population and who have intermarried frequently with Native Hawaiian, suggest that the risk in that population has changed very little over the last thirty years. The reason for this is unknown.

Figure 2.3.1 Female Breast Cancer: International comparisons-age-standardized rates (per 100,000 per year) 1988-1992



Source: Chia, et al, 2000

In addition, comparisons among population at low and high risk for beast cancer have noted significant difference in the shape of the age-specific incidence rates [Haenszel, et al 1973; Buell, et al 1973; Wynder, et al 1991]. Among Caucasian women, the increase in incidence rates levels off with age during the menopausal years, followed by a continued but slower rise during the postmenopausal years, whereas in Japan breast cancer incidence rates remain almost constant or even decline after menopause.

2.3.2 Ethnic diversity of breast cancer related risk factors

Ethnic variation in breast cancer is associated with many risk factors. Some of risk factors are related with modern lifestyle induced factors, such as not having children, oral contraceptive use, hormone replacement therapy, not breast-feeding, obesity and high-fat diets etc.

Brinton et al. [1997] observed a pattern of association for age at first full term pregnancy (FFTP) in black and white women ages 20-39 and 40-54 years in 1990-1991. Although late age at FFTP increased the risk for black women ages 20-39 years (comparing FFTP prior to age 20 years), no such increase was observed for the group ages 40-54 years. For white women, this pattern of association was almost reversed, with only a slight increase in risk with increasing age at FFTP for women ages 20-39 years and a substantially higher increase in risk for the group ages 40-54 years.

For total parity, Brinton et al. [1997] found a protective effect of high parity in both age subgroups of white women and only in the group of black women ages 40-54 years.

Frank D. et al [1998] conducted a population-based case-control study of breast cancer in Hispanic and non-Hispanic white women in New Mexico. They found that age at menarche, menopausal status, and age at menopause did not have strong influences on the risk of breast cancer among Hispanics or non-Hispanic whites, but they identified that parity, age at FFTP, and duration of lactation were independently associated with the risk of breast cancer in Hispanic women. The effect of parity and duration of lactation on the risk of breast cancer varied by ethnicity. Reproductive factors explained 17 percent of the ethnic difference in incidence for postmenopausal women and none of the difference among premenopausal women.

Few studies have focused on multicultural differences and the relation between hormonal risk factors and breast cancer. It is critical that these be done not only to provide information to promote public health in diverse population, but also to provide further insight into breast cancer etiology. It is likely that the metabolism of hormonal factors varies among populations according to genetic differences, and research into this possibility may help unravel the complicated puzzle of carcinogenesis. In this regard, further modeling of the crossover effect of breast carcinoma incidence among different ethnic women needs to be done. Risk factor profiles need to be compared and contrasted among the major ethnic population. These should include major endogenous hormonal risk factors, such as age at menarche and menopause, age at onset of regular menstrual cycles, effect of infertility, spontaneous or induced abortion, age at delivery, interval

between deliveries, parity and lactation duration. Exogenous hormonal use and its investigation are also critical and should include study of the use and duration of oral and parenteral contraceptives and postmenopausal HRT. In this regard, investigation of different hormonal formulations, their effects on circulating hormone levels, and their differential effects on risk in various populations is urgently needed. All of these studies should incorporate the field of molecular epidemiology, in hopes of discovering insights into the biologic mechanisms involved in observed epidemioglical risk factors among multicultural populations.

Chapter 3 Materials and Methods

The trends in breast cancer incidence were obtained from the published data (see below 3.1.2) and plotted according to the three different ethnic groups in Singapore: the Chinese, Malay and Indians. Various calculations and computations were then made (see below 3.1.3) in order to obtain standardized data and to make comparisons between the three ethnic groups. The annual percentage change in incidence rate was also calculated for the three different ethnic groups to investigate if there were any differences between the ethnic groups (See 3.1.3.2). The birth cohort effect was also plotted to investigate for difference between the three different ethnic groups (see 3.1.3.3).

Finally, a small pilot case-control questionnaire study was carried out in the oncology and medical outpatient clinics at the National University Hospital. The aim of the questionnaire was to study the possible risk factors of breast cancer in patients with breast cancer. Despite the small number acquired, such a study was still considered useful and important as a pilot study to suggest further more detailed studies.

3.1 Primary Study

3.1.1 Procedures for the registration of cancer in the Singapore Cancer Registry

Founded in 1967, the Singapore Cancer Registry is a national population based registry that is responsible for the collection, storage and analysis of basic clinical and

epidemiological data on all cancers diagnosed in Singapore. The registry is supported from the National University of Singapore, the Ministry of Health, the Singapore Cancer Society and the International Agency for Research on Cancer.

Registration is primarily based on notifications received from all sections of the medical profession in Singapore. To ensure that registrations are as complete as possible, the Registry routinely checks pathological records, hospital discharge records and death certificates (inclusive of private hospitals and laboratories). Cancer cases picked up from these sources are checked against previously registered cases. If the cases were not previously notified, reminders are sent to the doctors-in-charge for more information. Requests for clarification or additional information are also sent whenever necessary. When cases picked up from pathological records, hospital discharge records and death certificates are not notified by doctors, even after special request, they are registered by Registry staff provided they satisfy one of the following conditions, a pathological diagnosis of cancer, a clinical diagnosis of cancer supported by surgical, radiological or laboratory findings, or mention of cancer in the death certificate.

To ensure that the information provided by the Registry is of a high quality in terms of coverage and accuracy, a number of quality control checks are conducted. Registry staffs seek to rule out missed cases, duplicate notifications and inaccurate data. National Registration Identity Card (NRIC) number helps to ensure proper coverage and eliminate duplicate reporting effectively. In this way, close to complete coverage is ensured, as Singapore is a small country with good communication links.

3.1.2 Source of Data

The number of cases and incidence rates by age group of breast cancer were obtained from the Singapore Cancer Registry Report No.4 [Chia et al, 1996] and No. 5[Chia et al, 2000]. The Singapore Cancer Registry is a population-based registry covering the entire resident population of Singapore (Citizens and permanent residents). The population denominators in the reports were based on the censuses of 1970, 1980 and 1990, respectively. [Lau, 1992].

The Registry followed the international classification of Diseases for Oncology, 2nd Edition (ICD-0) (Report No.5) and 9th Revision (ICD-9) of international classification of Disease for the classification of Primary sites and morphology (Report No. 4).

3.1.3 Data Analysis

3.1.3.1 Truncated standardized incidence rate

To obtain the truncated standardized incidence rates, each five-year age group-specific incident rates were acquired from the Singapore Cancer Registry Report initially, and then they were adjusted for age using the "world" population by the Direct method (Waterhouse 1996), the expected incidence rates were calculated. Figures for world population were obtained from Cancer Incidence in Five Continents [Waterhouse, et al, 1993]. We combined the expected rates for the pre-menopause group to include 25 to 54 years old and the post-menopause group to include all from 55 years old and above, respectively. Table 3.1.1 showed the details. The methods of computation of truncated

standardized incidence rate of other each 5-year period by ethnicities were as same as that of 1968-1972, which allowed figures to be comparable between populations and over time.

Table 3.1.1 Example of computation of truncated standardized incidence rate of breast cancer. (Chinese, Malay and Indian women, Singapore, 1968-1972)

period	age	standardized-population	CHINESE	ExpectC	MALAY	expectM	INDIAN	expectI
1968-1972	25-	8000	2.8	0.224	5.1	0.408	5.8	0.464
1968-1972	30-	6000	11.1	0.666	8.3	0.498	15.2	0.912
1968-1972	35-	6000	21.4	1.284	27.7	1.662	12.8	0.768
1968-1972	40-	6000	36.8	2.208	24.1	1.446	46	2.76
1968-1972	45-	6000	46.2	2.772	44.3	2.658	33	1.98
1968-1972	50-	5000	60.7	3.035	45.2	2.26	35.2	1.76
total		37000		10.189		8.932		8.644
25-54y ASR				27.53784		24.14054		23.36216
1968-1972	55-	4000	62.2	2.488	58.9	2.356	70.5	2.82
1968-1972	60-	4000	63.9	2.556	50.4	2.016	44.4	1.776
1968-1972	65-	3000	66.3	1.989	78.1	2.343	67.9	2.037
1968-1972	70-	2000	59.8	1.196	29.1	0.582	247.2	4.944
1968-1972	75-	1000	45.1	0.451	67.5	0.675	0	0
1968-1972	80+	1000	59.9	0.599	0	0	483.1	4.831
total		15000		9.279		7.972		16.408
>=55y ASR				61.86		53.14667		109.3867

Data calculated from Singapore Cancer Registry Report No.4 & No.5 [Chia, et al 1996 &2000].

3.1.3.2 Annual percentage change in incidence rate

To obtain age-adjusted average annual percentage change for the estimation of trends across individual calendar years, data were analyzed by generalized linear regression model for discrete data.

When the number of cases occurring in different periods are known and if the logarithm of incidence varies linearly between two periods, the rate of change can be estimated by the slope of the line which best represents the logarithm of incidence as a linear function of period of diagnosis. The assessment of fit of this model was based on the Maximum Likelihood method.

The Poisson regression procedure uses an age-period model to calculate age-adjusted average annual percentage change according to the equation:

$$\text{Log (rate)} = \text{age}_i + b^* \text{ (period)}$$

With $i = 5,6\ldots16$, where there are 12 age groups (20-24, 25-29, …, >=75), and period was given as 1,2…6 (period 1 is 1968-1972, period 2 is 1973-1978 up to 1993-1997). This analysis was performed separately in different ethnic groups. The regression coefficient b is the increase in log of risk per five-year period. For example, if b=0.1620 (SE=0.001) for Chinese breast cancer, rate of change by 5-year period is the value of (e^b-1) or 0.1759. To obtain a time trend per year instead of per five-year period, we simply divide b by 5, getting 0.03518. We convert this trend to a relative risk of Exp(0.03518)=1.036, indicating an annual increase of 3.60%. To investigate the effect of ethnicity on breast cancer incidence rate, ethnic characteristic was added to the equation as covariate to calculate the relative risk and its likelihood-based 95% confidence interval. The calculations were carried out with the software SPSS, using a General Log linear Analysis. The analysis was performed separately by ethnicity.

3.1.3.3 Birth cohort plotting

In order to show the changes in time and inter-ethnic differences, the birth cohorts of age-specific incidence of breast cancer of each ethnic group were plotted. The data of age-specific rates were also used from the Singapore cancer registry. Age at diagnosis was adopted in the middle of the range in each five-year from 1968-1997, and the age-specific rates were obtained from those births between 1905 and 1950. All the data were shown as a series of longitudinal view rather than a series of cross-sectional view. Table 3.1.2 gives an example. The other two ethnic groups were plotted in the same way.

Table 3.1.2 Age-specific incidence rate of breast cancer in Chinese women arranged by birth cohort.

Date of Birth	Age											
	25y	30y	35y	40y	45y	50y	55y	60y	65y	70y	75y	80y
1905	__	__	__	__	__	__	__	__	66.3	86.9	90.4	97.9
1910	__	__	__	__	__	__	__	63.9	68.7	83.1	80.1	124.5
1915	__	__	__	__	__	__	62.2	80.1	79.5	93.6	117.5	114
1920	__	__	__	__	__	60.7	65.8	86.5	103.2	105.1	98.7	__
1925	__	__	__	__	46.2	53.5	72.9	76.3	124.9	115.6	__	__
1930	__	__	__	36.8	60.6	67	87.2	105.9	164.7	__	__	__
1935	__	__	21.4	36.6	71	81.9	100.6	117.5	__	__	__	__
1940	__	11.1	31.4	64.8	102	110.8	151.2	__	__	__	__	__
1945	2.8	11.1	37.6	60.9	125.2	149.3	__	__	__	__	__	__
1950	5.3	13.3	46.2	91.2	185.3	__	__	__	__	__	__	__

Data derived and calculated from Singapore Cancer Registry. [Chia, et al 1996 &2000].

3.2 Case-control study

The study was a hospital-based case-control study, which adopted incident cases and returning visit cases of breast cancer as cases and non-cancer patients visiting the same hospital as controls. All the consecutive cases and controls were recruited from National University Hospital (NUH) from May 1, 2001 to Feb 1, 2002. The case-control study comprised of 242 breast cases and 274 controls respectively. Based on ethnic difference, we separated the cases and controls into 3 groups: Chinese, Malay and Indian.

The risk factors were assessed by interview, using a multiple-choice questionnaire. The structured in-person interviews, which lasted a median of 25 minutes, collected detailed information regarding demographic factors, reproductive and menstrual history, contraceptive behavior, use of exogenous hormones, traditional medicine and screening history, anthropometrics and family history of cancer, alcohol consumption and smoking.

3.2.1 Data Collection

3.2.1.1 Selection of Cases

A case of breast cancer was defined as a histological confirmed carcinoma of the breast occurring in a Chinese/ Malay/ Indian female attending as outpatient or as an inpatient at the National University Hospital (NUH) from 1 May 2001 to 1 Feb 2002.

The criteria for eligibility of cases were female with primary carcinoma of the breast diagnosed in NUH (confirmed by screening mammography or biopsy), who were between the age of 18 and 75 years old, who lived in Singapore at least 5 years, who were mentally alert and coherent in their response to questions, and who gave consent for interview and tracing of records. Subjects who were subsequently determined to be suffering from cancer of another site, or who died from another cancer have not been included. Subjects who were not clearly diagnosed as primary breast cancer were also excluded.

Each subject was interviewed in the ward or at the specialist clinic (medicine, oncology, surgery) by the investigator, and a six-paged questionnaire was administered in person.

During the study period, completed interviews were obtained from 242 of the 276 eligible cases (87.7%) and 274 of the 284 eligible controls (96.5%). Reasons for non-interview included subjects refusing to provide interview information (4.8% in cases vs. 1.4% in controls) and serious illness (3.3% in cases vs. 1.8% in controls). Among cases, Chinese had slightly lower response rates than Malay or Indian subjects.

3.2.1.2 Selection of controls

Controls were selected from National University Hospital patients, frequency-matched by 10-year age group. For each case, one or more hospital controls were selected. Patients eligible to act as controls were Chinese/ Malay/ Indian females between 18 and 75 years of age. Controls who had disease in the breasts, gynaecological organs or endocrine

glands were excluded. Eligible controls were recruited into the study sequentially, from medicine and surgery specialist clinics.

3.2.1.3 Measurement of exposure

3.2.1.3.1 Study Instrument

A 6-paged questionnaire was developed and applied in a standardized manner to both cases and control subjects. Before administering the questionnaire, the purpose of the survey was explained to respondents, using the term "Women's health study" with no reference to breast cancer.

A copy of the questionnaire is attached in appendix. The questionnaire elicited information on demographic characteristics, menstrual history and reproductive history, oral contraceptive use and hormone replacement therapy history, socio-economical status, family history of cancer and benign breast disease history, smoking history and alcohol consumption history. Only the variables relevant to the present study will be described below.

Menopausal status was classified as premenopause, natural postmenopause, unnatural postmenopause, which including surgical (history of hysterectomy with or without oophorectomy) menopause, or chemical therapy menopause. Menopausal status was classified at the date of interview for controls, and at the date of cancer diagnosis for

cases. Women were classified as premenopausal if they had had a menstrual period within 1 year of the reference date and were not taking estrogens at the time.

A question on the outcome of pregnancy was also included. Women were considered to have "full-term delivered" only if the gestational at period was more than 36 weeks; if the gestational period was more than 28 weeks and less than 36 weeks, this was considered to be a "premature delivered baby"; spontaneous abortion was considered "miscarriage", medical or surgery induced abortion was considered "abortion".

For the question of current body weight and height, the same investigator throughout the whole study period did measurements. Body weight (kg) in light clothes and height (cm) without shoes were determined to the nearest one decimal point using the SECA Balance with a height attachment. Body Mass Index (BMI) was then calculated using the formula:

BMI= weight (kg) ÷ Height (m) ^{2}.

BMI of less than 18.5 kg/m^2 was taken as underweight,

18.5 kg/m^2 to 24.9 kg/m^2 as healthy weight range,

25.0 kg/m^2 or more as overweight.

Respondents were classified as having a history of benign breast disease if they gave a positive answer when asked if they had "ever had" the disease. A positive family history of cancer was defined as a "yes" response to "has anyone in your family ever had cancer?" and if that person was a first-degree relative, or relative other than husband/ husband's family.

3.2.1.3.2 Techniques to reduce bias

It is widely accepted that one of the chief sources of systematic differences in the case-control study is that arising from bias in measuring exposure. In order to minimize this, it was considered important that the conduct of the interview, and the responses elicited should not be influenced by knowledge of disease status. As far as possible, cases were interviewed soon after admission (if in-patients) and before being called to see the doctor (if out-patients).

Since recall bias can lead to either an over or underestimation of the association between exposure and disease, during our investigation, one of the most common methods of gathering information was by interview either of the study subjects themselves and their surrogates, such as spouses of participants or sisters of affected patients. However, it is thought that women who have breast cancer were more likely to be truthful about the fact that they had some risk factors, such as induced abortion, compared to women who do not have breast cancer.

A second type of systematic error in collecting information is interview bias, which refer to any systematic difference in the soliciting, recording, or interpreting of information from study participants. Since in a case-control study, knowledge of a subject's disease status may cause differential probing by the questioner for previous exposure history, this may cause bias regarding exposure history. Consequently, prevention of bias in the design phase of the investigation is crucial to the validity of the study results; it means that highly objective, closed-ended questions for subjects were very important. In addition, since

information concerning a subject's exposure status was available at the time and disease status was determined, investigators who were aware of the study hypothesis may be more or less likely to record the outcome of interest for individuals known to have the exposure under examination, the questionnaire we provided contains mainly questions that are not open and could be completed by subjects themselves. In the case of questions that need some explanation (A pilot study had been done before the case-control study), we furnished clear and detailed definition of some exposures in the protocol (See 3.2.1.3).

Another source of bias in the study concerns the potential for loss of subjects to follow-up due to some reason. Even though it is not so important for our case-control study as that in a cohort study, any observed association would be biased if persons are lost to follow-up. As mentioned, patients were therefore followed up till the diagnosis was verified. If the diagnosis could not be verified by the time of ending of the study (1 Feb 2002), the data was used to check for measurement bias only, and was not used in the final analysis.

For this study, one control with a history of breast cancer was removed from the analysis. In addition, 3 cases that indicated on interview that they did not have the minimal 5-year residency in Singapore were also eliminated from analysis. To reduce effects of detection bias, we eliminated 12 cases diagnosed as breast cancer combined with cervical, uterus or ovarian cancer.

With respect to the administration of the data collection instrument, the single most important way to minimize the potential for bias was to maintain blindness to the greatest possible extent. In practical terms, in order to blind subjects, we named our questionnaire

"Women's health research", which means, subjects would be unaware of an individual's disease status when assessing exposure in this study.

3.2.1.3.3 Control of confounding factors in the design and analysis

Confounding involves the possibility that the observed association is totally or in part due to the effects of differences between the study groups other than the exposure under study that could affect their risk of developing the outcome of interest. Since confounding can lead to an overestimate or underestimate of the true association between exposure and disease and can even change the direction of the observed effect, it is very important to control confounding in two phases of the questionnaire: design and analysis.

a. Methods to control confounding in the design

Confounding cannot occur if the potential confounding factors do not vary across either the exposure or the disease categories. One way to achieve this is to restrict the admissibility criteria for subjects and limit entrance into the study to individuals who fall within a specified category or categories of the confounder. For example, in our study, as race was a potential confounding factor; the study could include only Chinese, Malay and Indian women, and excluded completely other races. In addition, obesity was another potential confounding factor, and since some endocrine disease were associated with obesity, hence, we excluded those women who had diabetes in our subjects.

b. Methods to control confounding in the analysis

Stratification is a technique to control confounding in the analysis of a study that involves the evaluation of the association within homogeneous categories or strata of the confounding variable. For example, as ethnicity is a potential confounder, an estimate of the association between the exposure and disease would be calculated for Chinese, Malay and Indian women separately. Each of these stratum-specific estimates is, by definition, unconfounded by ethnic, since there is no variability of the confounding variable within the stratum.

Multivariate analysis allows the efficient estimation of measures of association while controlling for a number of confounding factors simultaneously even in situations where stratification would fail because of insufficient numbers. In general, a multivariate technique refers to any analysis of data that takes into account a number of variables simultaneously. The most common way that many factors are controlled for simultaneously is through the use of a multiple regression model. In our study, the outcome of interest was a binary variable such as breast cancer versus non-breast cancer. In such circumstances, it was possible to use a specialized type of logistic regression analysis, which was a powerful statistical tool for estimating the magnitude of the association between an exposure and a binary outcome after adjusting simultaneously for a number of potential confounding factors. This model is a simple variant of the multiple regression equation, in which the risk of developing an outcome is expressed as a function of independent predictor variables.

3.2.2 Data Analysis

Bivariate odds ratios (OR) for risk of breast cancer and 95% confidence intervals (95% CI) were calculated for ethnic status, and for each of the variables of interest (Breslow and Day 1980). P value of 0.05 or less was accepted as significant. Mean values (±S.D.) were analyzed by ethnicity and age group. Logistic regression analysis was then employed using Enter approach to control potential confounding factors, and to select the significant independent variables (covariates). This was done by computing Odds ratio [Benichou and Gail; 1990] and associated 95% Confidence Intervals. Procedures from the SPSS software package version 10.0 (SPSS Inc., Chicago, IL) were used.

Chapter 4 Results

The results are presented in three parts below. In sections 4.1 and 4.2, the observed data illustrates the significant increasing trend of breast cancer in Singapore over the past 30 years and the different patterns of increase among Chinese, Malay and Indian groups separately. In the section 4.3, the case-control study displays the magnitude and differences of some known and identified risk factors in subjects by ethnic group.

4.1 Breast cancer incidence trend in Singapore

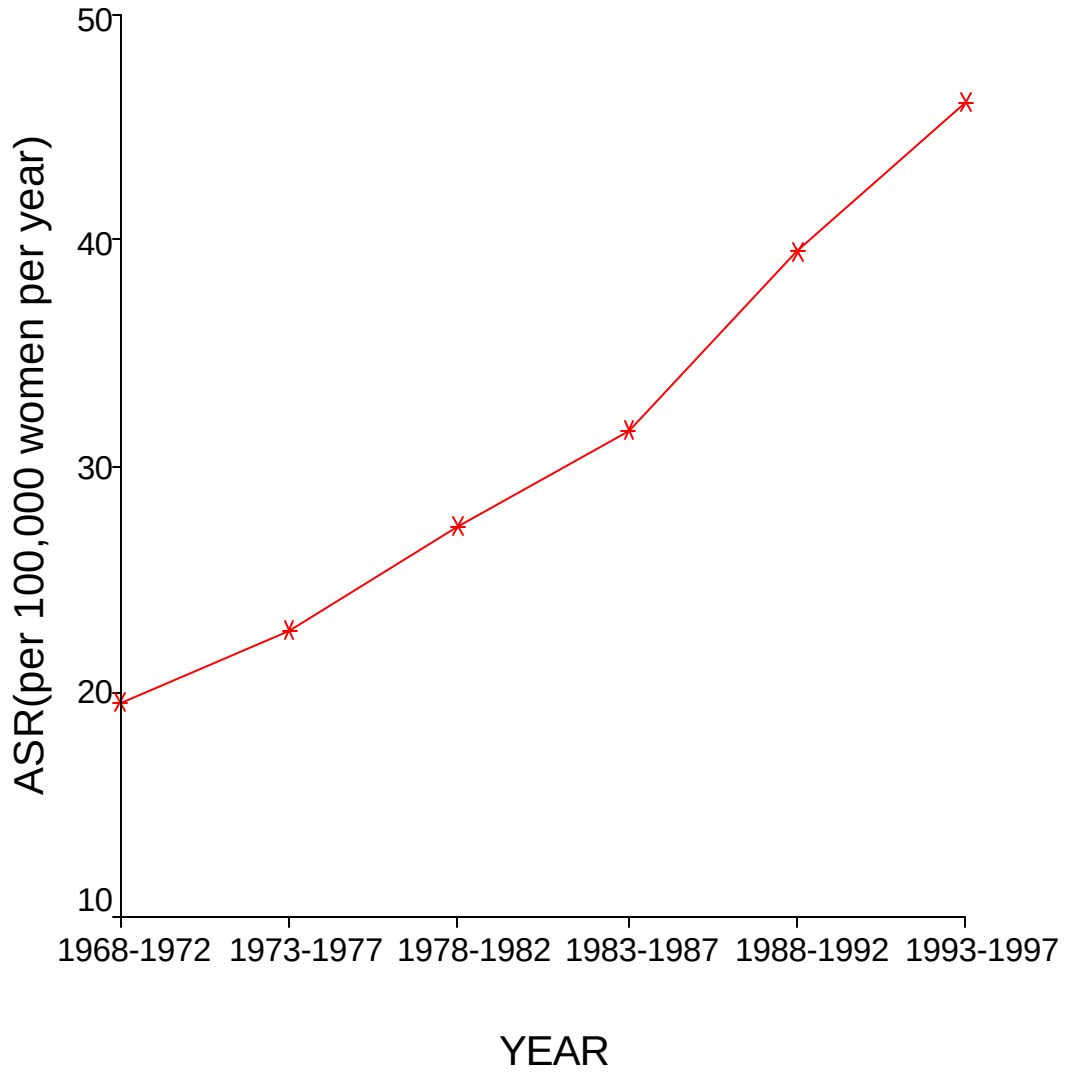
In Singapore, the absolute annual incidence number of medically certified breast cancer patients showed an increasing trend in the 30 years from 1968 to 1997. The age-adjusted incidence rate per 100,000 also showed an increase from 19.5 per 100,000 women in 1968-1972 to 46.1 per 100,000 women in 1993-1997 (Fig 4.1.1). This age-adjusted incidence rate of breast cancer in 1993-1997 is 2.3 times than that in 1968-1972. There has been a consistent increase over time across all age-specific groups.

Figure 4.1.2 displays the age-adjusted rates for each 5-year period of all Residents of Singapore women. It can be seen from the figure that there is an increasing pattern across each age-specific group.

The bimodal age distribution of Singapore female subjects with breast carcinoma can be observed in each 5-year period line. The two peaks are in the 45 to 49 years age group and

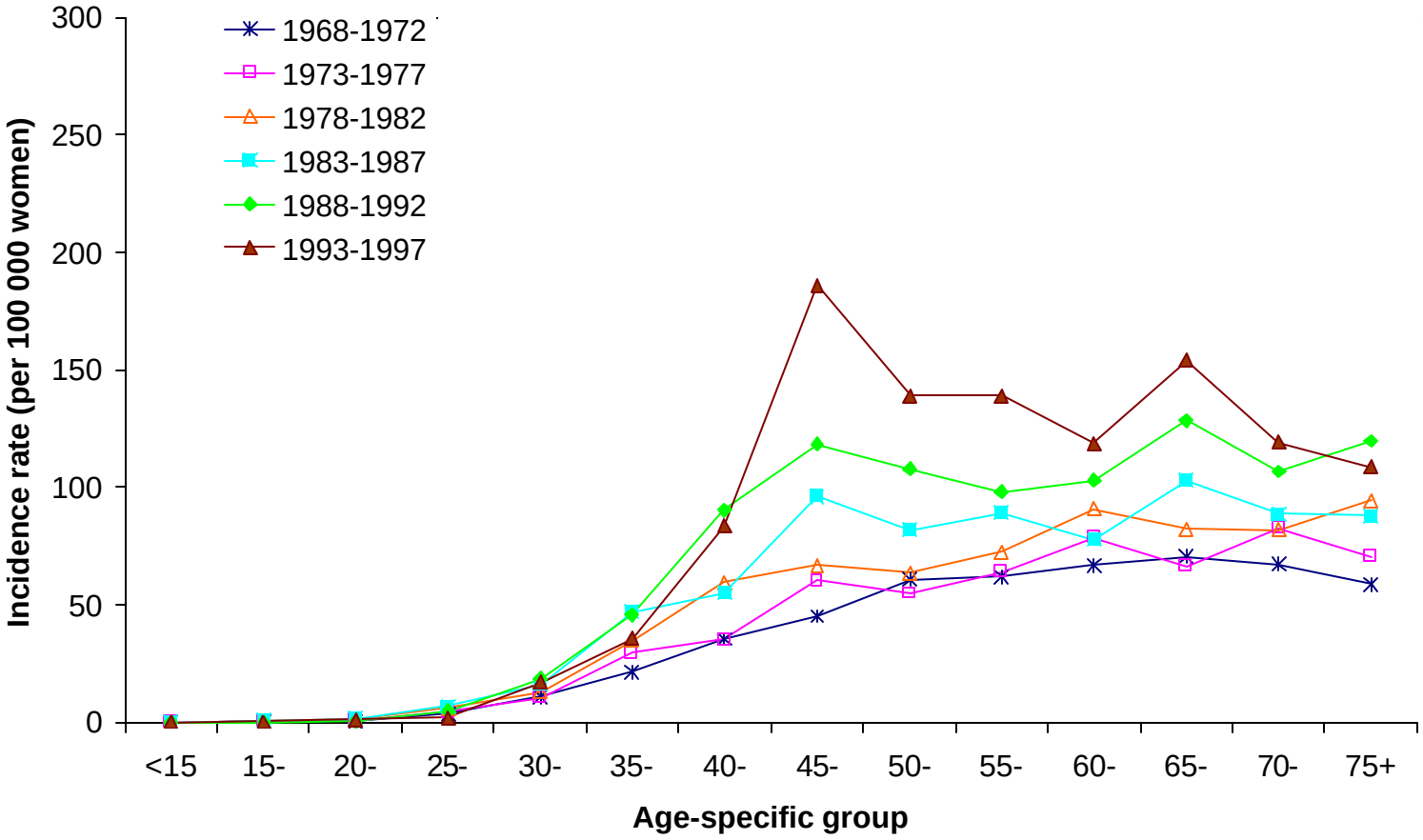
at about 65 years age. This graph has the same character as in other Asian countries, such as China and Japan [Tao, et al, 1988], with a tendency to display a “plateau” between 50 to 60 years. After 60 years of age, the age-specific breast cancer risk increases again.

Figure 4.1.1 All Female Residents Incidence Rate of Breast Cancer in Singapore (1968-1997)



Source: Singapore Cancer Registry (ASR: Age standardized incidence rate)

Figure 4.1.2 Age-specific Incidence Rate of Breast Cancer, All Residents of Singapore (1968-1997)



4.2 Ethnic difference incidence Rate of breast cancer among three ethnic groups

Chinese, Malay and Indian are the three main ethnic groups in Singapore. In the population, the proportion of the three ethnic groups is 77.7%, 14.1%, and 7.1% respectively (1990 census). From the data of the Singapore Cancer Registry, it can be noticed that while there are increasing trends present in all three ethnic groups, there are some different characteristics between the three groups.

The number of medically certified incidence of breast cancer was the highest among the Chinese, followed by the Malays and Indians. However, when compared with the total female population of all races in Singapore, there was no statistically significant difference in the percentage of Chinese having breast cancer (Table 4.2.1).

From Table 4.2.2, it can be seen that all three ethnic groups show an increase in incidence of breast cancer over the past three decades. The greatest increase was in the Malay group, followed by Chinese group. Indian women have the lowest rate of increase, although the small number of cases makes interpretation of the overall trend difficult.

In Singapore, the incidence in 1988-1997 in Chinese (43.3 per 100,000) and Malay women (37.6 per 100,000) appeared to be higher than Indian women (34.3 per 100,000). Indian women thus have a lower incidence of breast cancer compared with the other two main ethnic groups in Singapore in the last decade.

Table 4.2.1 Number of cases (age-standardized incidence) of breast cancer by ethnic group

Ethnic group	Period					
	1968-1972	**1973-1977**	**1978-1982**	**1983-1987**	**1988-1992**	**1993-1997**
Chinese	553 (19.5)	740 (22.7)	1050 (27.4)	1450 (31.6)	2188 (39.5)	2984 (47.1)
Malay	63 (16.9)	70 (15.3)	111 (21.1)	153 (23.1)	261 (33.9)	354 (41.2)
Indian	25 (25.1)	41 (27.5)	52 (29.9)	86 (33.1)	111 (31.9)	169 (36.8)
All female Resident	670 (19.9)	863 (22.1)	1237 (26.8)	1724 (31.0)	2609 (38.7)	3574 (46.1)

Source: Singapore cancer registry (Chia, et al, 1996& 2000)

Table 4.2.2 Average annual percentage changes in incidence by ethnic group

Ethnic Group	Average annual Change	95% CI
All groups	3.5%	3.1%, 3.9%
Chinese	3.6%	3.3%, 3.9%
Malay	4.0%	2.3%, 5.8%
Indians	1.4%	0.9%, 2.0%

Source: Singapore cancer registry (Chia, et al, 1996& 2000)

4.2.1 Age and race incidence

Between 1993-1997, the median age at presentation in Chinese women is 52.5 years with mean age 53.58 ± 0.23 years compared with a median age of 47.5 years in Malay women with mean age 49.86± 0.66 years and a median age of 52.5 years in Indian women with mean age 53.7± 1.00 years. This shows a similarity in median age in the two ethnic groups of Chinese and Indian women at the age of presentation, while Malay women have a statistically significantly lower median age ($P<0.05$, t-test). 60% of Chinese women are less than 55 years compared with 67.4% of Malay women and 53.8 % of Indian women. The two differences suggest that Malay women have breast cancer at a younger age than that in Chinese and Indian women. It also can be seen that the median age of breast cancer in all three ethnic groups of Singapore women are much younger than that in Western women whose the median age is 60-64 years [Kelsey, et al, 1993]. Studies in Japan and China also show a younger mean and median age of presentation in these countries [Tao, et al, 1988]. These suggest that breast cancer presents at a younger age in Asians.

4.2.1.1 Trend and age incidence

In Fig 4.2.1, the incidence rate increases till about 47 years old in Chinese women. The period with highest rate is 45-50 years. The rates then tend to decrease in the older age groups. There are two peaks in breast cancer incidence in Chinese women. The highest peak is at 47 years, and the second peak is at 65-70 years old.

Figure 4.2.1 Trends of breast cancer in Chinese women in Singapore, 1968-1997

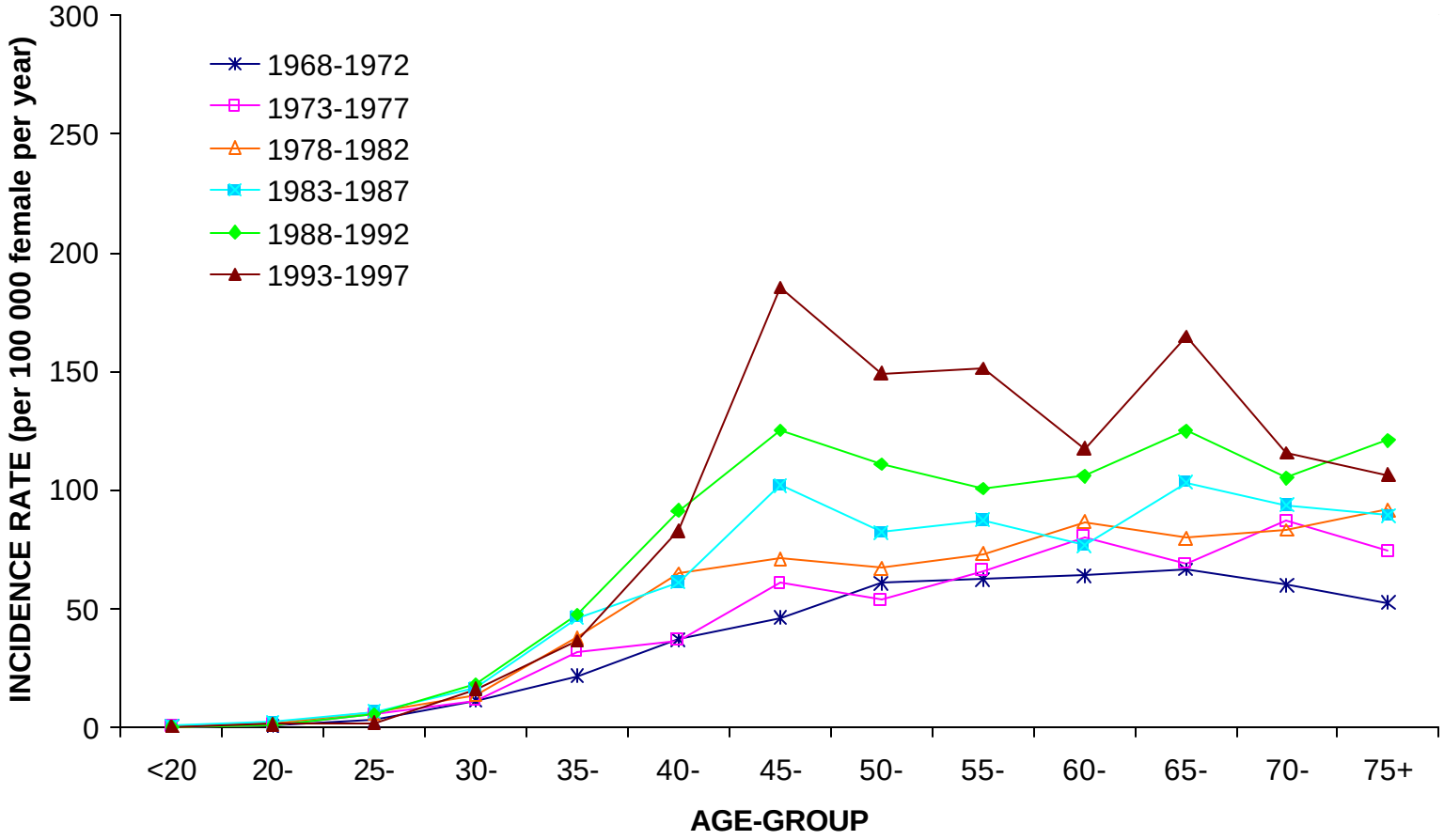


Figure 4.2.2 Trends of breast cancer in Malay women in Singapore, 1968-1997

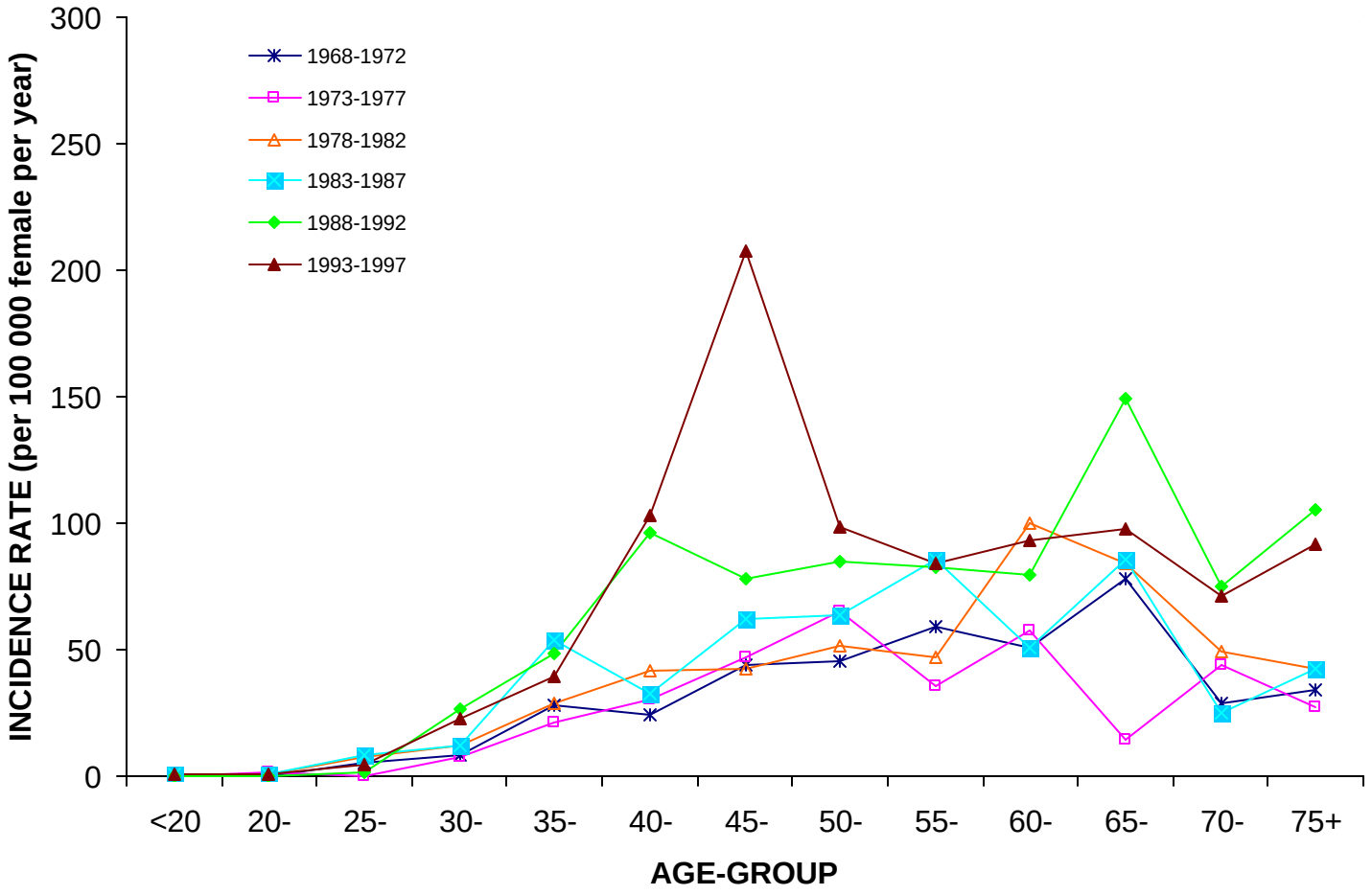
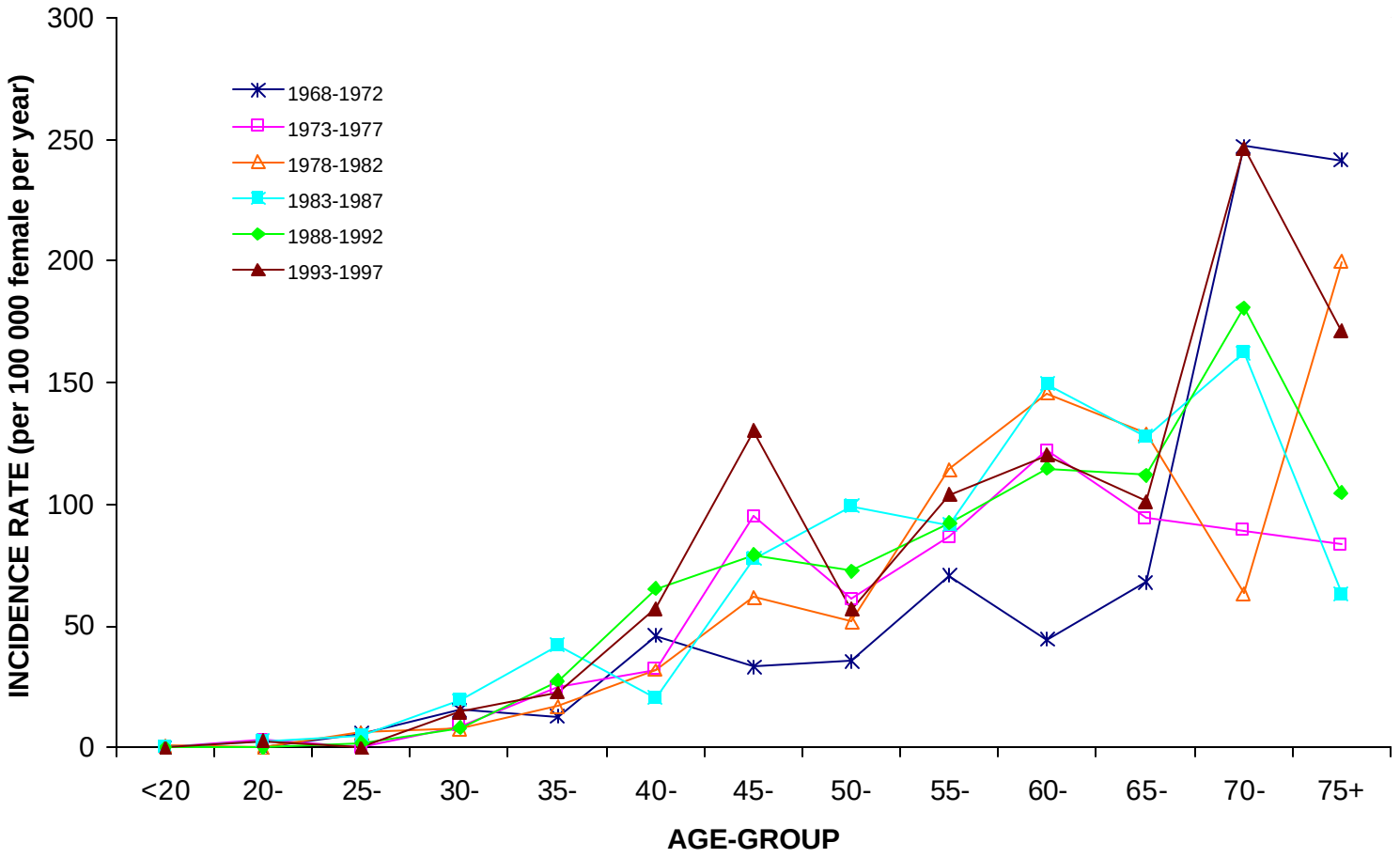


Figure 4.2.3 Trends of breast cancer in Indian women in Singapore, 1968-1997



As Fig 4.2.2 shows, Malay women show a difference in the change in incidence rate, the increasing trend is not similar to that of the Chinese group. Each line only has one peak. In addition, the peak in Malay group is different in different lines. This may be partly due to the smaller denominator of Malay women, but the consistency of the "double-peak" in Chinese women is not present.

In Figure 4.2.3, the difference in Indian women is also clear: the highest peaks are after 65 years old in all the incidence rates.

4.2.1.2 Menopause status and ethnic incidence

The Chinese group had the highest incidence rate in premenopausal women. In contrast, the Indian incidence rate was the highest in the postmenopausal women.

To further investigate the relationship of menopause to the incidence of breast cancer, we divided the risk population into two groups, the pre-menopause group: 25 to 54 years old and the post menopause group 55 years old and older.

Fig 4.2.4 shows an increasing trend in the incidence rate in all three ethnic groups. This is to say, in the younger than 55 years old women, or pre-menopause women, incidence rates of breast cancer increased with time. The Chinese had the highest incidence rates, followed by Malays and Indians. However, the pre-menopause Indian women group has an obvious difference in the lower increase in breast cancer incidence compared to the Malay and Chinese groups.

Figure 4.2.4 Breast cancer incidence rate of different ethnic group in Singapore 1968-1997(25-54y)

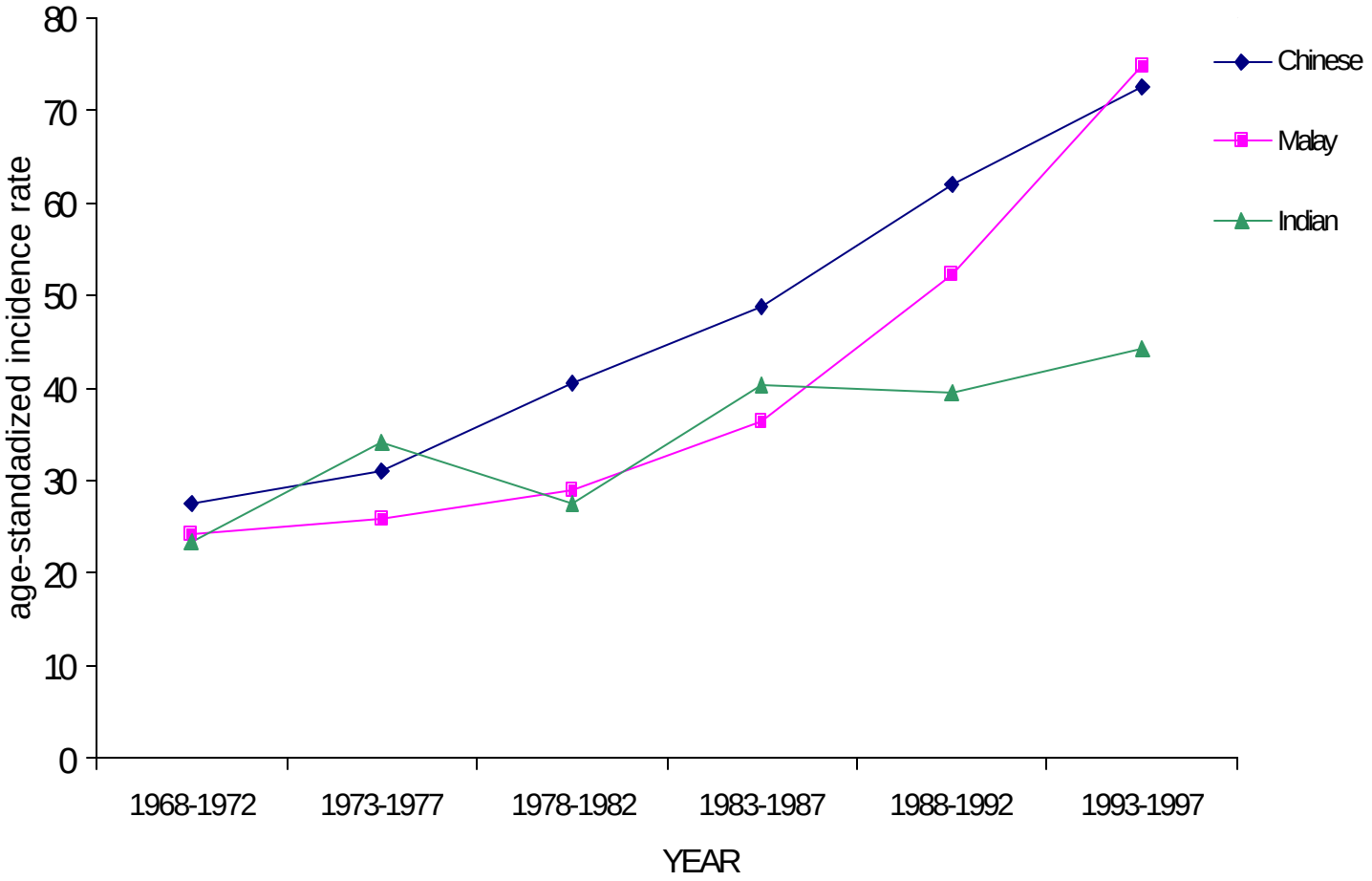


Figure 4.2.5 Breast cancer incidence rate of different ethnic group in Singapore 1968-1997 (55-75^{+}y)

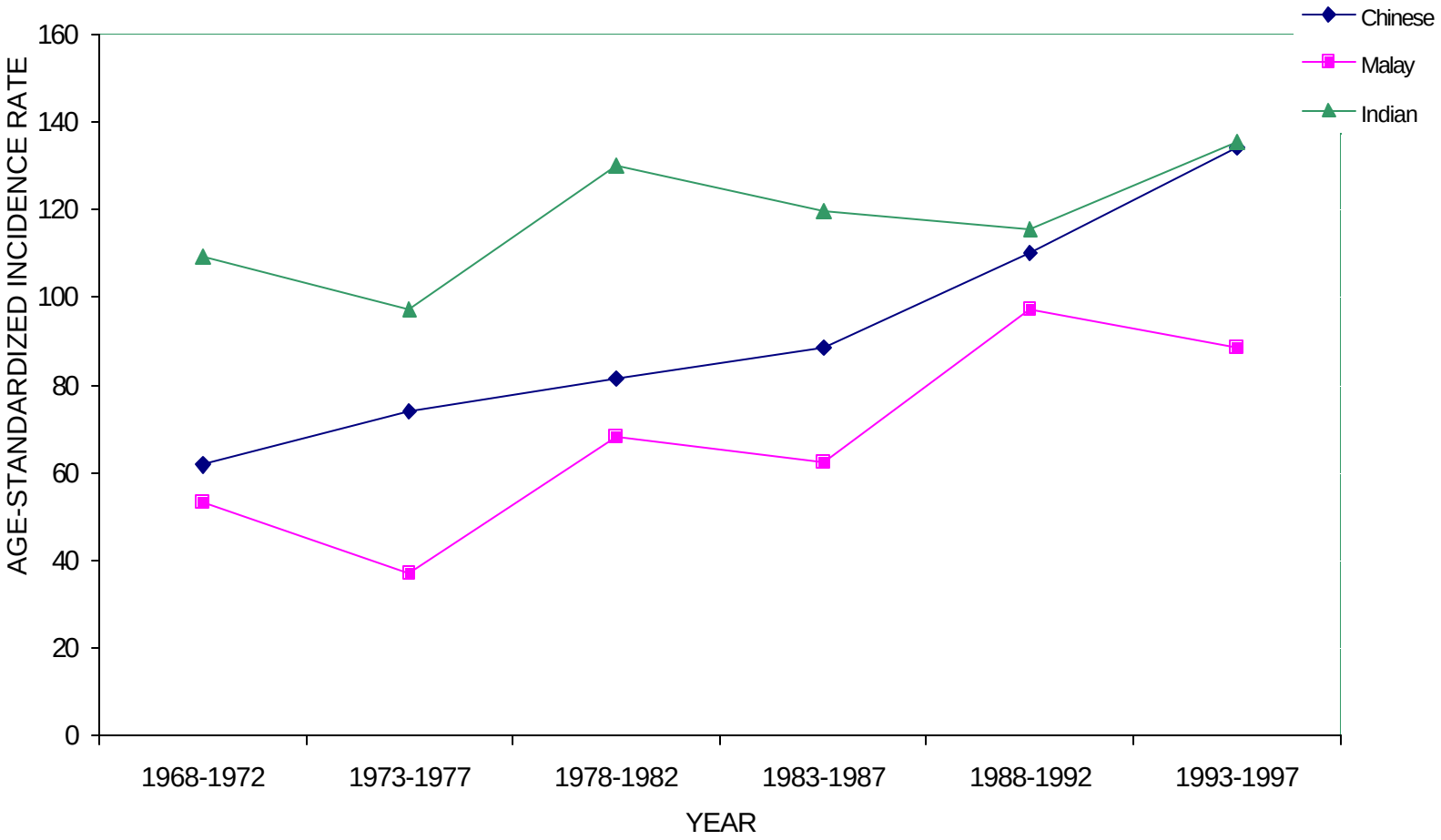


Fig 4.2.5 shows a contrasting trend in the 55-year and older age compared to the pre-menopausal group. The Indians have the highest incidence and the Chinese group has gradually and recently increased to catch up with similar incidence rates of the Indian post-menopausal group.

4.2.1.3 Birth cohort effect

Figure 4.2.6-4.2.8 are plotted to show the age-specific incidence of breast cancer by birth cohort from 1968-1997 in three main ethnic groups in Singapore.

The figures represent the data as a series of longitudinal studies rather than before, a series of cross-sectional studies. This longitudinal view of the data is also emphasized on a linear scale in a plot of the incidence rates of breast cancer against age for each birth cohort.

When the age-specific trends are plotted according to cohort year of birth, breast cancer risk in the Chinese group appears to increase among the older women born after 1905 (Figure 4.2.6). Between births in 1905-1930, there is a slow rise, and then a sharp increase among women born thereafter. There is a tendency for successive birth cohorts to have higher age-specific rates compared with previous cohorts, suggesting a strong cohort effect.

The striking differences in the Indian women group suggest some protection against the environmental factors that have caused the increase in breast cancer incidence in the Malay and Chinese group. In Figure 4.2.7 and Figure 4.2.8, because of the smaller

denominator of Malays and Indians, the higher variability makes the trends more difficult to interpret.

Figure 4.2.6 Age-specific incidence of breast cancer by birth cohort. Singapore Chinese 1968-1997

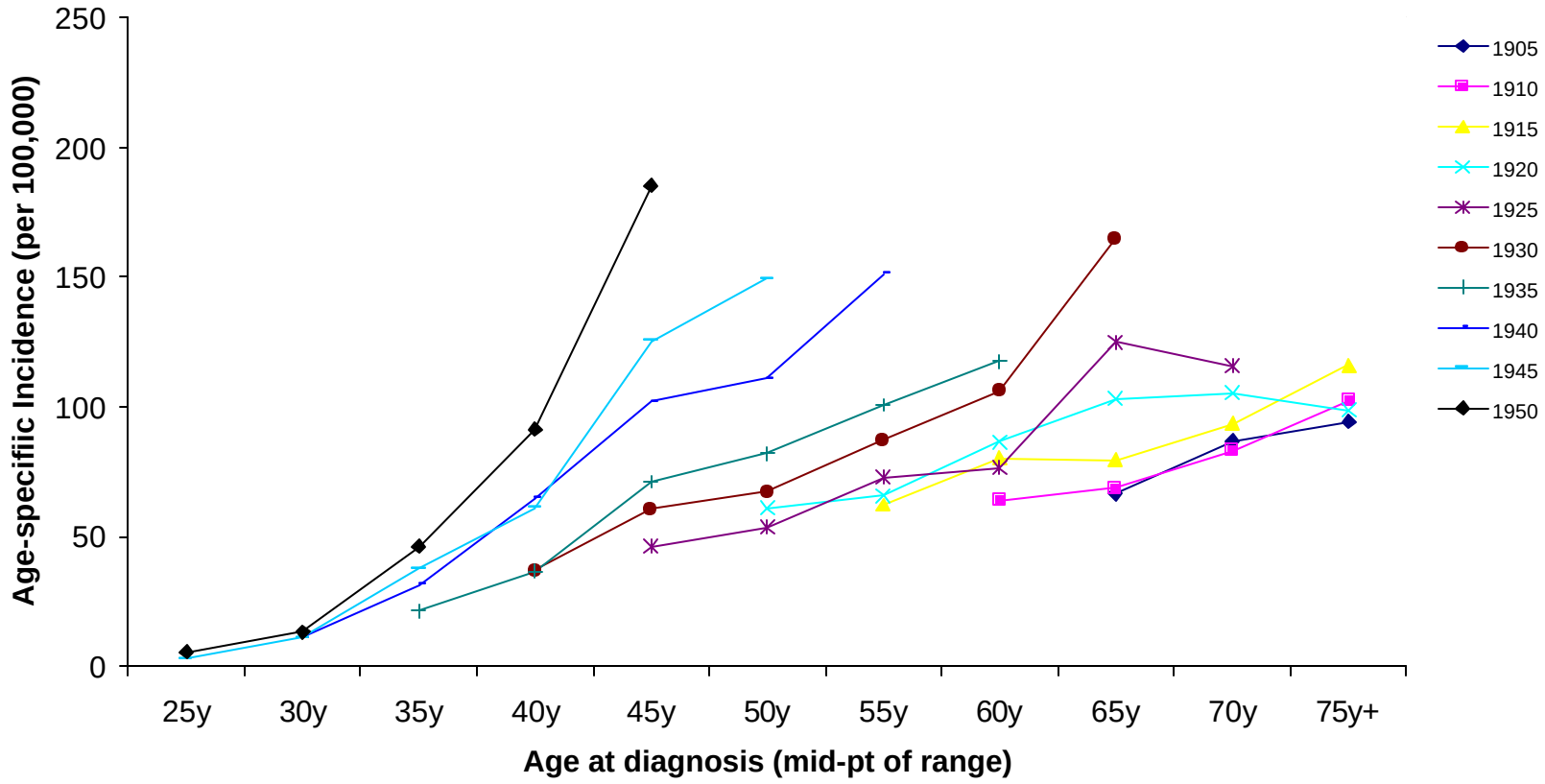


Figure 4.2.7 Age-specific incidence of breast cancer by birth cohort. Singapore Malay 1968-1997

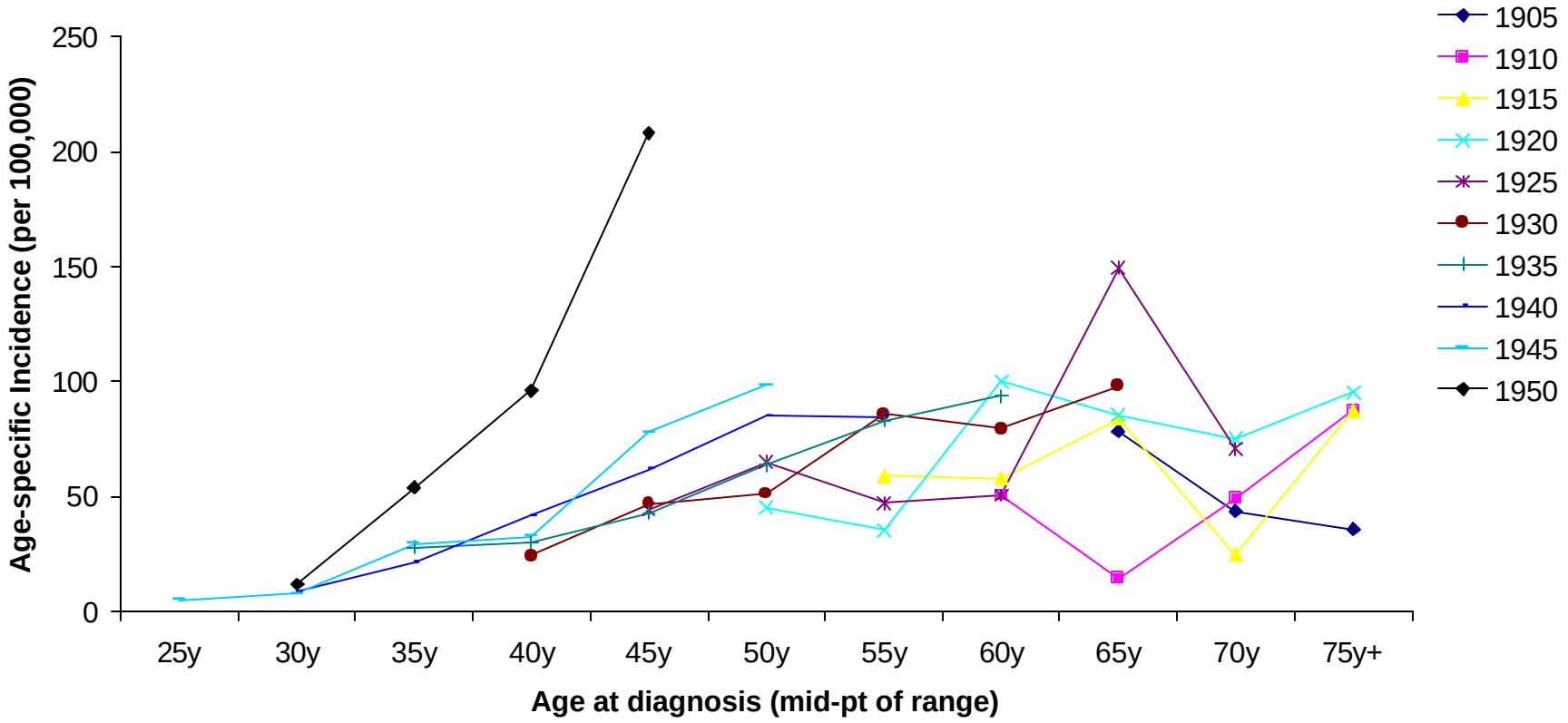
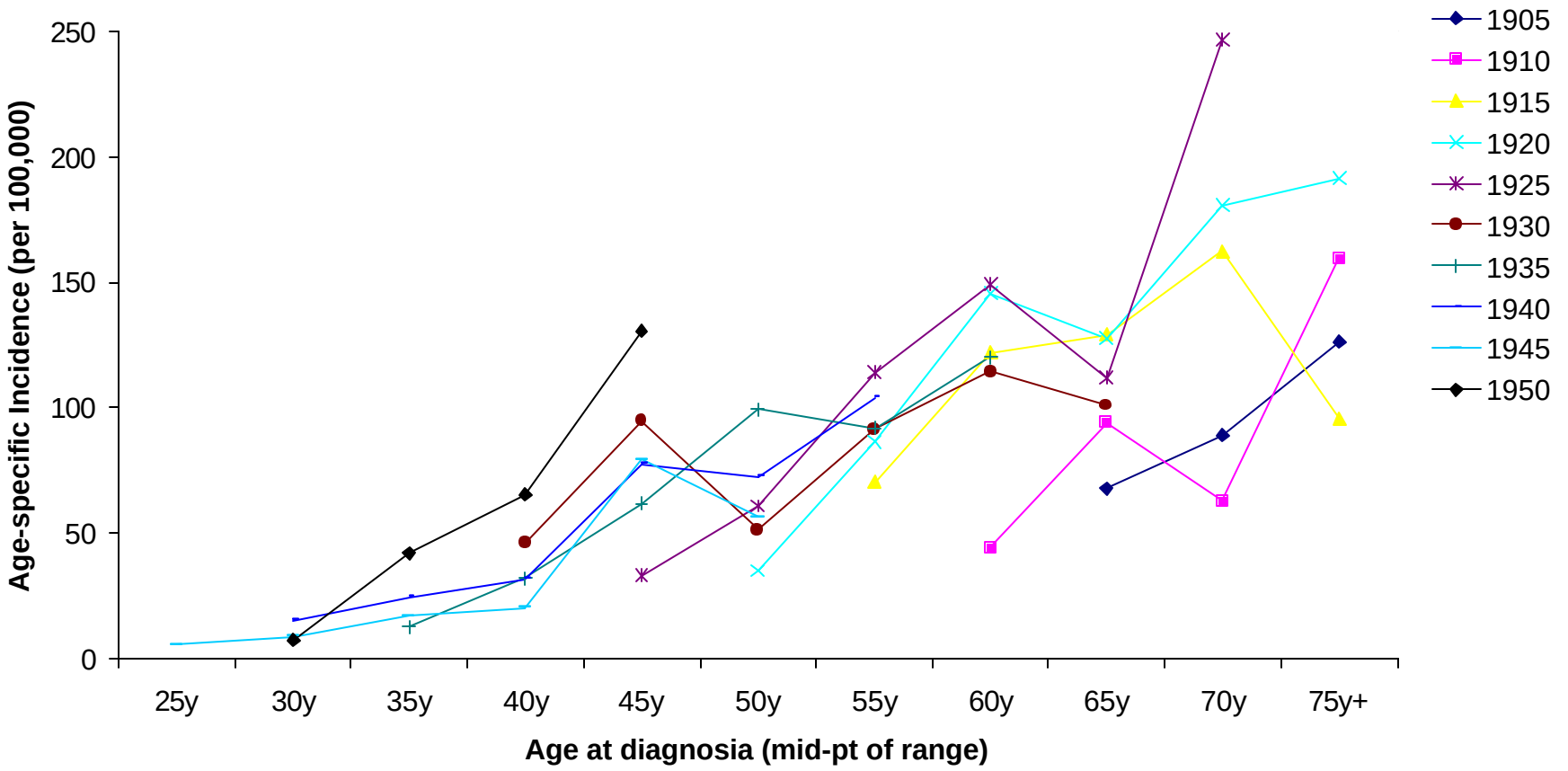


Figure 4.2.8 Age-specific incidence of breast cancer by birth cohort. Singapore Indian 1968-1997



4.3 Case-control study

4.3.1 General information

The case-control study comprised of a total of 242 patients with histologically confirmed breast cancer from the National University Hospital, and 274 hospital control subjects of the same age distribution as the cases. Not all patients (or controls) agreed to be interviewed fully. Reasons for non-participation by ethnicity and case-control status are shown in the table 4.3.1. Refusal rates for Chinese women were higher than for Malay and Indian women (8.1% vs. 6.6% vs. 8%). Women with breast cancer were more likely to refuse to participate than their control counterparts (total of 12% vs. 3.1%).

We compared the age matched Chinese, Malay and Indian control subjects to identify and verify expected racial differences in specific variables. The results of the comparisons are indicated in Figure 4.3.1. We found that Indian and Malay women had early menarche and earlier age of first full-term pregnancy than Chinese. Surprisingly, the mean age of last regular menstrual period of Indian women (46 years) was significantly younger than Chinese (51 years) and Malay women (50 years) ($P<0.05$, t-test). We are not aware of any previous reports of this difference in age of menopause.

Table 4.3.1 Reasons for nonparticipation by ethnicity and case-control status

	Chinese		Malay		Indian	
	Cases	Controls	Cases	Controls	Cases	Controls
Total eligible participants	208 (100%)	214 (100%)	37 (100%)	38 (100%)	31 (100%)	32 (100%)
Interview complete	182 (87.5%)	206 (96.3%)	33 (89.2%)	37 (97.4%)	27 (87.1%)	31 (96.9%)
Subject refusal	8 (3.8%)	4 (1.9%)	1 (2.7%)	0 0	1 (3.2%)	0 0
Serious illness	5 (2.4%)	3 (1.4%)	2 (5.4%)	1 (2.6%)	2 (6.5)	1 (3.1%)
Others*	13 (6.3%)	1 (0.5%)	1 (2.7%)	0 0	1 (3.2%)	0

Others* includes 12 cases who were diagnosed as breast cancer combined with cervical, uterus or ovary cancer, 3 cases that did not have at least 5-year residency in Singapore and 1 control with a history of breast cancer.

Figure 4.3.1 Characteristics of control subjects by ethnicity

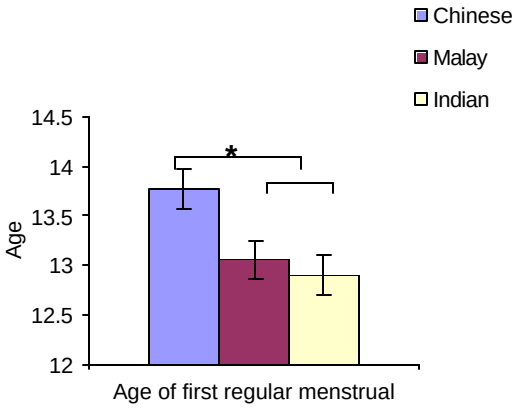


Figure 4.3.1.a

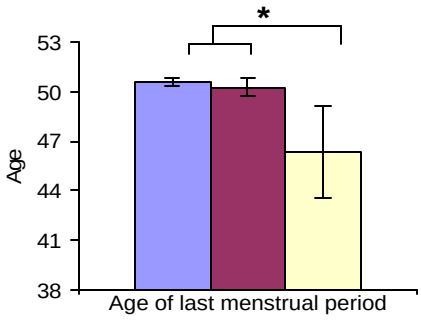


Figure 4.3.1.b

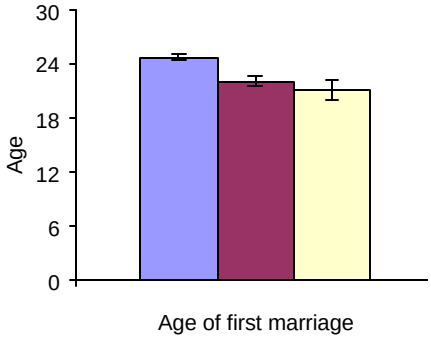


Figure 4.3.1.c

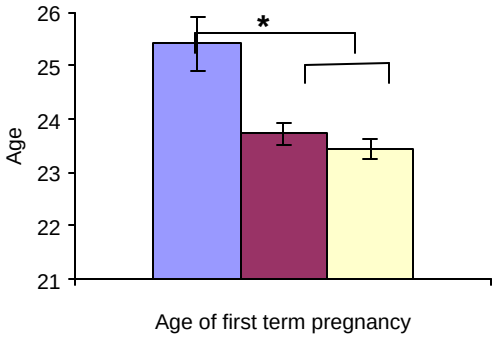


Figure 4.3.1.d

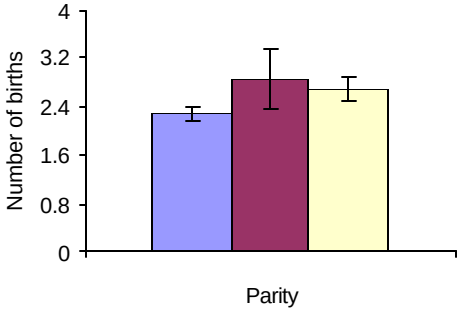


Figure 4.3.1.e

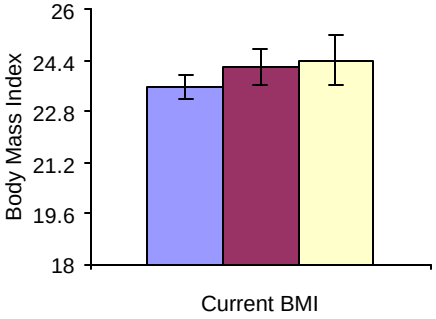


Figure 4.3.1.f

* P<0.05

4.3.2 Socio-demographic characteristics of the study population

The major socio-demographic characteristics of the study population (breast cancer patients and controls) are described in Table 4.3.2. In the breast cancer patients, the age at presentation in Malay women was significantly younger ($P<0.01$), with a median age of 45.4 years compared with a median age of 53.1 years in Chinese and 54.3 years in Indians.

There was no significant difference between cases and controls in the distribution of completed education years in the Chinese group. But in the Malay and Indian groups, compared with controls, breast cancer patients were more likely to have completed 6-9 years (72.7% vs. 43.2% and 55.6% vs. 38.7% respectively) of full time education.

The proportion of living in =5-room house was highest in Chinese compared to Indian and Malay (total subjects in each ethnic group). Compared with controls, there was no significant difference between cases and controls about living in =3 room house in each ethnic group. For other social economic factors, such as family car ownership, breast cancer patients were more likely to own a car, but no statistically significant differences can be seen in each ethnic group ($P>0.05$).

In addition, because of the very small number of women who drink alcohol or smoke, no significant difference was observed between Chinese, Malay and Indian breast cancer patients. Thus these two items do not feature as risk factors in Singapore compared to Western countries.

Table 4.3.2 Socio-demographic characteristics of breast cancer and controls among Singapore women, 2000-2001

Characteristic	Percentage of distribution of cases and controls (Chinese women)		Percentage of distribution of cases and controls (Malay women)		Percentage of distribution of cases and controls (Indian women)	
	Cases (n=182)	Controls (n=206)	Cases (n=33)	Controls (n=37)	Cases (n=27)	Controls (n=31)
Age at interview (years)						
<30	0.5(1)	1.5(3)				
30-39	3.8(7)	4.4(9)	6.1(2)	5.4(2)	7.4(2)	6.5(2)
40-49	15.9(29)	17.0(35)	42.4(14)	43.2(16)	18.5(5)	22.6(7)
50-59	47.8(87)	46.1(95)	45.5(15)	43.2(16)	25.9(7)	29.0(9)
60-69	18.1(33)	18.4(38)	6.1(2)	8.1(3)	29.6 (8)	25.8(8)
>=70	13.7(25)	12.6(26)			18.5(5)	16.1(5)
Mean (SD)	53.1(10.9)	52.5(11)	45.4(6.5)	45.7(6.7)	54.3(12.9)	53.4(12.1)
[Total] Mean	52.8 (10.9)		45.6 (6.5)		53.8 (12.4)	
P value	0.64		0.81		0.51	
Marital Status						
Married	86.3 (157)	93.2 (192)	81.8 (27)	100.0(37)	92.6 (25)	90.3 (28)
Unmarried	13.7(25)	6.8(14)	18.2(6)	0	7.4(2)	9.7(3)
P value	0.03*				0.87	
Education (years completed)						
=<5	32.4(59)	31.1(64)	24.2(8)	51.4(19)	33.3(9)	32.3(10)
6~9	46.2(84)	49.5(102)	72.7(24)	43.2(16)	55.6(15)	38.7(12)
10~15	20.9(38)	18.9(39)	3.0(1)	5.4(2)	3.7(1)	25.8(8)
>=16	0.5(1)	0.5(1)			7.4(2)	3.2(1)
P value	0.57		0.06		0.69	

Table 4.3.2. (Continued)

Characteristic	Percentage of distribution of cases and controls (Chinese women)		Percentage of distribution of cases and controls (Malay women)		Percentage of distribution of cases and controls (Indian women)	
	Cases (n=182)	Controls (n=206)	Cases (n=33)	Controls (n=37)	Cases (n=27)	Controls (n=31)
Dwelling						
=<3-room	24.7(45)	23.8(49)	24.2((8)	29.7(11)	29.6(8)	25.8(8)
4-room house	25.3(46)	26.2(54)	54.5(18)	37.8(14)	25.9(7)	38.7(12)
>=5-room	50.0(91)	50.0(103)	21.2(7)	32.4(12)	44.4(12)	35.5(11)
P value	0.54		0.75		0.9	
Own a car						
No	61.0(111)	66.5(137)	72.7(24)	86.5(32)	59.3(16)	74.2(23)
Yes	39.0(71)	33.5(69)	27.3(9)	13.5(5)	40.7(11)	25.8(8)
P value	0.29		0.23		0.27	
Alcoholic drinking						
Never	91.8(167)	91.7(189)	97.0(32)	100.0(37)	88.9(24)	83.9(26)
Yes	8.2(15)	8.3(17)	3.0(1)	0	11.1(3)	16.1(5)
P value	0.99				0.71	
Cigarette smoking						
Never	94.5(172)	94.2(194)	87.9(29)	100.0(37)	96.3(26)	93.5(29)
Yes	5.5(10)	5.8(12)	12.1(4)	0	3.7(1)	6.5(2)
P value	0.89				0.64	

*P value less than 0.05

4.3.3 Hormonal and reproductive related factors in different ethnic groups

Table 4.3.3 shows the comparative results for the racial differences that include 9 hormonal and reproductive related variables of primary interest. Multivariate odds ratios and 95 percent confidence intervals were computed for Chinese, Malay and Indian women, and adjusted by age, education, dwelling, family car ownership, smoking and alcoholic drinking. The variances of the relative risk estimates are greater for Malay and Indian women because of the smaller sample size.

Generally, women with breast cancer were more likely to have a lower parity (P=0.08), older age at first full term pregnancy (P=0.06) or null pregnancy (P=0.08) compared with control women.

In the distribution of menstrual cycle length (days), a shorter menstrual cycle of less than 28 days was one risk factor compared with 29-32 days in Chinese group (OR 1.99, 95% C.I. 1.29~3.07, P<0.05) and Indian group (OR 488, 95% C.I. 1.60~14.86, P<0.05), but this was not significant in Malay group, even though the relative risk was 1.35 (95% C.I 0.47~3.89). There were no significant relationships between age at menarche and breast cancer in our subjects, even through the odds ratio of age at menarche younger than 12 years old was 1.46 (95% C.I. 0.90~2.36) in Chinese group and 1.40 (95% C.I. 0.38~5.26) in Indian group.

It can be observed that the relative risk of breast cancer among Chinese decreased with younger age at first marriage, and increased in unmarried women. The protective factor of an age of marriage less than 20 years old was statistically significant (OR 0.41, 95% C.I. 0.21~0.81, P<0.05). However, when we compared across the 3 ethnic groups of breast cancer patients this item, we found that 27.3% of Malay and 40.7% of Indian married at the age of younger than 20 years, while only 9.3% of Chinese married at that age (P<0.05).

In the distribution of age at first pregnancy (Table 4.3.3.a) (limited to women with children), an age of first pregnancy of older than 30 years old was a significant risk factor in Chinese women (OR 2.42, 95% C.I 1.32~4.42, P<0.05). By contrast, in the Indian group, an age of first pregnancy of younger than 20 years old decreased the risk of breast cancer (OR 0.16, 95% C.I. 0.04~0.70, P<0.05).). In addition, it should be noticed that 25% of Chinese breast cancer patients were older than 30 years old when they experienced the first pregnancy, while the percentage of Malay and Indian were 4.0% and 9.1%.

A similar pattern of association for Chinese and Malay began to emerge for such factors as number of full-term pregnancies (Table 4.3.3.b) (analysis limited to married women) and total months of breast-feeding (Table 4.3.3). In the Chinese group, compared to women with 1 to 2 life births, women with 3~4 life births had a lower risk of breast cancer (OR 0.52, 95% C.I. 0.31~0.86, P<0.05). An even greater protective effect was observed in those who had more than 5 life births (OR 0.37, 95% C.I 0.14~0.97, P<0.05). Although the Malay group had a similar trend, the statistically significance was not observed,

probably due to the limited sample size. For total months of breast-feeding, a dose-response effect also was seen in the Chinese group, while a general overall protective effect was seen in other two ethnic groups, but the small sample sizes of the Indian and Malay groups gave a non-significant dose-response effect. However, we still can see some differences among these three ethnic groups in these items. Only 31.2% of Chinese breast cancer patients have more than 3 children, while the percentage in Malay and Indian are 55.5% and 52% respectively. In contrast, more than half of Chinese patients (64.3%) have null breast-feeding, while only 30.3% of Malay and 40.7% of Indian did not ever breast feed.

The relative risk of breast cancer among Chinese did not increase with increasing months of oral contraceptive use. But it indicated a higher risk of breast cancer in cases of "ever used" OC compared with the "never used" group. This was also observed in the Malay group.

When we limited the regression to women who had a natural menopause (Table 4.3.3.c), the age of menopause did not show any statistically significant difference between control women and breast cancer women in the Chinese or Malay groups. But in the Indian group, controls' menopause age (46 years) was significantly younger than breast cancer patients (53 years). The adjusted odds ratio was 1.45 (95% C.I. 1.07~1.97, $P<0.05$).

When we restricted the logistic regression analyses to parous Chinese women, the results (See Table 4.3.3.d) indicated that age at first time abortion and number of abortions or miscarriages were associated with breast cancer risk in Chinese women (Since so few

Malay or Indian women admitted to abortion, no further analysis was carried out in these two groups). The results were indicative of a larger relative risk in those who had their first time abortion or miscarriage at an older age of greater than 30 years old. The adjusted odds ratio was 4.58 (95% C.I. 2.25~9.35) compared with those who never had those experiences. In addition, regardless of the age of abortion or miscarriage, there were higher risks in the group that experienced one or more times abortion or miscarriages. The adjusted odds ratio was 2.54 (95% C.I. 1.60~4.02) compared with those who didn't.

Table 4.3.3 Adjusted odds ratio and 95% confidence intervals for breast cancer by female hormonal and reproductive related factors among Chinese, Malay and Indian women in Singapore, 2001-2002

	Percentage of distribution of cases and controls (Chinese women)		Adjusted odds ratio (95% C.I.)	Percentage of distribution of cases and controls (Malay women)		Adjusted odds ratio (95% C.I.)	Percentage of distribution of cases and controls (Indian women)		Adjusted odds ratio (95% C.I.)
	Cases (n=182)	Controls (n=206)		Cases (n=33)	Controls (n=37)		Cases (n=27)	Controls (n=31)	
Cycle length (days) of menarche									
29~32	47.8 (87)	59.7 (123)	1	66.7 (22)	64.9 (24)	1	29.0 (8)	66.7 (21)	1
=<28	47.8 (87)	32 (66)	1.99* (1.29~3.07)	33.3 (11)	24.3 (9)	1.35 (0.47~3.89)	71.0 (19)	33.3 (10)	4.88* (1.60~14.86)
>=32	4.4 (8)	8.3 (17)	0.68 (0.28~1.65)	0	10.8 (4)		0	0	
Age at menarche									
>=14y	45.1 (82)	49.0 (101)	1	51.5 (17)	35.1 (13)	1	22.2 (6)	32.3 (10)	1
13y	23.1 (42)	26.7 (55)	0.96 (0.580~1.6)	15.2 (5)	24.3 (9)	0.44 (0.12~1.62)	29.6 (8)	12.9 (4)	3.57 (0.72~17.76)
=<12y	31.9 (58)	24.3 (50)	1.46 (0.90~2.36)	33.3 (11)	40.5 (15)	0.53 (0.17~1.61)	48.1 (13)	54.8 (17)	1.40 (0.38~5.26)
Age at first marriage									
20~30y	66.5 (121)	66.5 (137)	1	45.5 (15)	64.9 (24)	1	40.7 (11)	29.0 (9)	1
Unmarried	13.7 (25)	6.8 (14)	2.43* (1.18~5.02)	18.2 (6)	0		7.4 (2)	9.7 (3)	0.54 (0.07~3.97)
=<20	9.3 (17)	18.0 (37)	0.41* (0.21~0.81)	27.3 (9)	32.4 (12)	1.20 (0.41~3.53)	40.7 (11)	51.6 (16)	0.55 (0.17~1.78)
>30	10.4 (19)	8.7 (18)	1.21 (0.60~2.42)	9.1 (3)	2.7 (1)	4.81 (0.45~51.42)	11.1 (3)	9.7 (3)	0.85 (0.14~5.31)
Total months of breast feeding									
0	64.3 (117)	42.7 88)	1	30.3 (10)	10.8 (4)	1	40.7 (11)	19.4 (6)	1
=<4	23.6 (43)	18.4 (38)	0.82 (0.49~1.38)	24.2 (8)	27.0 (10)	0.32 (0.07~1.40)	11.1 (3)	22.6 (7)	0.23 (0.04~1.25)
4~15	7.1 (13)	23.3 (48)	0.21* (0.10~0.40)	21.2 (7)	48.6 (18)	0.16* (0.04~0.68)	22.2 (6)	45.2 (14)	0.23* (0.06~0.93)
>=16	4.9 (9)	15.5 (32)	0.18* (0.08~0.41)	24.2 (8)	13.5 (5)	0.69 (0.13~3.65)	25.9 (7)	12.9 (4)	0.94 (0.18~4.78)
Take medicine disrupted menarche (oral contraceptive/ estrogen pill/ traditi onal Chinese medicine) (months)									
0	77.5 (141)	91.3 (188)	1	63.6 (21)	94.6 (35)	1	88.9 (24)	83.9 (26)	1
=<6	7.1 (13)	1.9 (4)	4.32* (1.38~13.5)	9.1 (3)	0		0	6.5 (2)	
7~12	3.3 (6)	1.5 (3)	2.66 (0.65~10.8)	0	0		7.4 (2)	6.5 (2)	1.08 (0.14~8.37)
>12	12.1 (22)	5.3 (11)	2.65* (1.23~5.70)	27.3 (9)	5.4 (2)	7.56* (1.49~38.47)	3.7 (1)	3.2 (1)	1.08 (0.06~19.03)
Take hormone									
Never	77.5 (141)	95.1 (196)	1	87.9 (29)	100.0 37)	1	88.9 (24)	83.9 (26)	1
Yes	22.5 (41)	4.9 (10)	5.68* (2.75~11.7)	12.1 (4)	0		11.1 (3)	16.1 (5)	0.65 (0.14~3.02)

Table 4.3.3.a

	Percentage of distribution of cases and controls (Chinese women)		Adjusted odds ratio (95% C.I.)	Percentage of distribution of cases and controls (Malay women)		Adjusted odds ratio (95% C.I.)	Percentage of distribution of cases and controls (Indian women)		Adjusted odds ratio (95% C.I.)
	Cases (n=148)	Controls (n=175)		Cases (n=25)	Controls (n=37)		Cases (n=22)	Controls (n=28)	
Age at first pregnancy (Limited to with children)									
20~30y	66.2 (98)	73.1 (128)	1	60.0 (15)	70.3 (26)	1	77.3 (17)	39.3 (11)	1
=<20y	8.8 (13)	15.4 (27)	0.56 (0.26~1.17)	36.0 (9)	24.3 (9)	1.73 (0.56~5.32)	13.6 (3)	42.9 (12)	0.16* (0.04~0.70)
>30y	25.0 (37)	11.4 (20)	2.42* (1.32~4.42)	4.0 (1)	5.4 (2)	0.85 (0.07~10.55)	9.1 (2)	17.9 (5)	0.27 (0.04~1.64)

Table 4.3.3.b

	Percentage of distribution of cases and controls (Chinese women)		Adjusted odds ratio (95% C.I.)	Percentage of distribution of cases and controls (Malay women)		Adjusted odds ratio (95% C.I.)	Percentage of distribution of cases and controls (Indian women)		Adjusted odds ratio (95% C.I.)
	Cases (n=157)	Controls (n=192))		Cases (n=33)	Controls (n=37)		Cases (n=25)	Controls (n=28)	
No. Of full term pregnancies (limited to married women)									
1~2	62.4 (98)	49 (94)	1	37.0 (10)	37.8 (14)	1	36.0 (9)	39.3 (11)	1
0	6.4 (10)	8.9 (17)	0.58 (0.25~1.34)	7.4 (2)	0		12.0 (3)	0	
3~4	24.8 (39)	33.9 (65)	0.52* (0.31~0.86)	48.1 (13)	51.4 (19)	0.94 (0.31~2.80)	44.0 (11)	53.6 (15)	0.87 (0.25~3.0)
>=5	6.4 (10)	8.3 (16)	0.37* (0.14~0.97)	7.4 (2)	10.8 (4)	0.67 (0.10~4.59)	8.0 (2)	7.1 (2)	1.14 (0.11~12.28)

Table 4.3.3.c

Menopausal age (limited to natural postmenopause women)	Chinese		Malay		Indian	
	Mean age	Odds ratio	Mean age	Odds ratio	Mean age	Odds ratio
Control	50.57	1	50.25	1	46.33	1
Case	51.08	1.0 (0.91~1.1)	51.20	1.38 (0.72~2.65)	52.77	1.45* (1.07~1.97)

*P value less than 0.05

Table 4.3.3.d

Abortion information related with breast cancer (Chinese)

	Cases	Controls	Odds Ratio
	N (%)	N (%)	
Times of abortion or miscarriage (Married Women only)			
None	88(56.1%)	147(76.6%)	1
>=1	69(43.9%)	45(23.4%)	2.54* (1.60~4.02)
Age of first time abortion or miscarriage (Married Women only)			
None	88(56.1%)	146(76.0%)	1
<20y	2(1.3%)	1(0.5%)	3.23 (0.29~36.16)
20-30y	33(21.0%)	33(17.2%)	1.67 (0.96~2.90)
>=30y	34(21.7%)	12(6.3%)	4.58* (2.25~9.35)

*P value less than 0.05

Adjusted for age (years), education (years of schooling), marital status (married or not), dwelling (classified by types of house), whether own a car, smoking and alcoholic drinking by logistic regression analysis.

4.3.4 Anthropometrics in different ethnic groups

Table 4.3.4 compares the anthropometric measurements that of the three ethnic groups in our study. Adjusted odds ratio (by age, education, marital status, dwelling, family car ownership, smoking and alcoholic drinking) and 95% confidence intervals for Chinese, Malay and Indian women were computed.

No differences were seen in the age-adjusted associations with breast cancer among Chinese, Malay and Indian women for height at age 18 years. Even through the odds ratio of a height greater than 150cm is greater than 1 among three ethnic groups, there was no statistically significant relationship between breast cancer risk and taller women in our study population.

Surprisingly, we observed an increased risk of breast cancer in the Chinese group when the body mass index at age 18 years was less than 18.5kg/m^2. The odds ratio is 1.92 (95% C.I. 1.23~2.9) ($P<0.05$). In the Malay and Indian groups, again the sample size was too small to study such an effect. In addition, there are no differences in BMI (at 18 years old) distributions among the three ethnic groups. These results are surprising and it is difficult to reconcile with the hypothesis of body mass index with breast cancer. There are probably many other as yet clinical and molecular factors relevant in this group to be further investigated.

In the Malay and Indian groups, the proportion of heavier body mass index as adults was higher in breast cancer patient group than in control group. We found that a current higher body mass index was associated with an increased risk of breast cancer among Malay women. The odds ratio was 3.9 (95% 1.39~11.2) ($P<0.05$). It should be noted that the number of Malay women in this category was small. However, the percentage of higher BMI (more than 25kg/m2 current) in Indian breast cancer patients (63%) is similar as that of Malay (60.6%), which is strikingly higher than that of Chinese (27.6%).

Table 4.3.4 Adjusted odds ratio and 95% confidence intervals for breast cancer by anthropometric factors among Chinese, Malay and Indian women in Singapore, 2001-2002

	Percentage of distribution of cases and controls (Chinese women)		Adjusted odds ratio	Percentage of distribution of cases and controls (Malay women)		Adjusted odds ratio	Percentage of distribution of cases and controls (Indian women)		Adjusted odds ratio
	Cases (n=182)	Controls (n=206)		Cases (n=33)	Controls (n=37)		Cases (n=27)	Controls (n=31)	
Height at age 18y (cm)									
<150	14.8 (27)	16.0 (33)	1	21.2 (7)	27.0 (10)	1	3.7 (1)	12.9 (4)	1
150~164	75.3 (137)	74.3 (153)	1.11 (0.64~1.9)	75.8 (25)	70.3 (26)	1.40 (0.46~4.28)	88.9 (24)	71.0 (22)	4.68 (0.47~46.40)
>=164	9.9 (18)	9.7 (20)	1.17 (0.51~2.7)	3.0 (1)	2.7 (1)	1.34 (0.07~26.1)	7.4 (2)	16.1 (5)	1.77 (0.11~28.58)
Body mass index (kg/m2) at age 18y									
18.5~24.9	60.4 (110)	73.3 (151)	1	53.1 (17)	51.4 (19)	1	55.6 (15)	51.6 (16)	1
<18.5	37.4 (68)	24.3 (50)	1.92* (1.23~2.99)	40.6 (13)	35.1 (13)	1.12 (0.39~3.17)	37.0 (10)	38.7 (12)	0.87 (0.28~2.63)
>=25	2.2 (4)	2.4 (5)	1.13 (0.29~4.3)	6.3 (2)	13.5 (5)	0.44 (0.07~2.7)	7.4 (2)	9.7 (3)	0.73 (0.11~5.0)
Body mass index (kg/m2) current									
18.5~24.9	66.3 (120)	55.5 (111)	1	27.3 (9)	62.2 (23)	1	37.0 (10)	58.1 (18)	1
<18.5	6.1 (11)	10.5 (21)	0.49 (0.23~1.08)	12.1 (4)	2.7 (1)	10.05 (0.95~106)	0	0	2.39 (0.83~6.9)
>=25	27.6 (50)	34.0 (68)	0.67 (0.43~1.05)	60.6 (20)	35.1 (13)	3.94* (1.39~11.2)	63.0 (17)	41.9 (13)	1.0 (0.97~1.05)

*P value less than 0.05

Limit BMI at age 18y to premenopause women and BMI current to postmenopause women.

Adjusted for age (years), education (years of schooling), marital status (married or not), dwelling (classified by types of house), family car ownership, smoking and alcoholic drinking by logistic regression analysis.

4.3.5 Family history and benign breast disease history in different ethnic groups

The relationship between family history or benign breast disease and breast cancer risk among Chinese, Malay and Indian subjects are described in Table 4.3.5, adjusted for confounding factors by age, education, marital status, dwelling, family car ownership, smoking and alcohol drinking.

A history of benign breast disease was associated with breast cancer risk among all three ethnic groups, but the confidence intervals indicated statistical significance only in Chinese 5.67 (95% C.I. 2.95~10.91) ($P<0.05$) and Indian groups 4.03 (95% C.I. 1.08~14.99) ($P<0.01$).

Since the number of patients with a positive family history of breast cancer was too small to compute, we combined all items of reported family members (excluding spouse or spouse's family) with a past history of cancer. The adjusted odds ratio associated with positive family history was 1.78 (95% C.I. 1.16~2.71) ($P<0.05$) in the Chinese group. Although in Malay or Indian groups, there was also a higher odds ratio, the relationship of positive family history and breast cancer was not statistically significant.

Table 4.3.5 Adjusted odds ratio and 95% confidence intervals for breast cancer by female family history and benign breast disease among Chinese, Malay and Indian women in Singapore, 2001-2002

	Percentage of distribution of cases and controls (Chinese women)		Adjusted odds ratio	Percentage of distribution of cases and controls (Malay women)		Adjusted odds ratio	Percentage of distribution of cases and controls (Indian women)		Adjusted odds ratio
	Cases (n=182)	Controls (n=206)		Cases (n=33)	Controls (n=37)		Cases (n=27)	Controls (n=31)	
History of benign breast disease									
No	73.1 (133)	93.7 (193)	1	87.9 (29)	91.9 (34)	1	63.0 (17)	87.1 (27)	1
Yes	26.9 (49)	6.3 (13)	5.67* (2.95~10.91)	12.1 (4)	8.1 (3)	1.58 (0.33~7.70)	37.0 (17)	12.9 (4)	4.03** (1.08~14.99)
Family history of cancer									
No	58.2 (106)	71.4 (147)	1	63.6 (21)	78.4 (29)	1	55.6 (15)	74.2 (23)	1
Yes	41.8 (76)	28.6 (59)	1.78* (1.16~2.71)	36.4 (12)	21.6 (8)	2.16 (0.70~6.61)	44.4 (12)	25.8 (8)	2.39 (0.78~7.33)

*P value less than 0.05;

** P value less than 0.01.

Adjusted for age (years), education (years of schooling), marital status (married or not), dwelling (classified by types of house), family car ownership, smoking and alcoholic drinking by logistic regression analysis.

Chapter 5 Discussion and Conclusion

The primary objective of this study was to investigate aspects of breast cancer in Singapore. Particularly, our intention was to investigate the ethnic variation of breast cancer in Singaporean women in the past three decades and to determine the possible reasons for the different pattern of breast cancer among the Chinese, Malay and Indian population. We also conducted a hospital-based case-control study matched for ethnic group and age to obtain information on known and identified risk factors by personal interview.

5.1 Different patterns of breast cancer among Chinese, Malay and Indian groups

As the incidence of breast cancer is known to vary among countries or ethnic groups, [Kelsey, et al, 1993; Miller, et al, 1986], the present study has shown that ethnic variation also exists in Singapore. Different patterns of increase in breast cancer incidence among Chinese, Malay and Indian have been shown in the present study.

On the whole, all three ethnic groups show increases in the incidence of breast cancer over the past 3 decades. The greatest incidence is in the Chinese group while the highest annual increase is shown in the Malay group. There is a strong suggestion of a bimodal distribution in age-specific incidence, with the two peaks at 45 and at 65 years seen clearly in the Chinese group. In addition, the Indian group has the highest post-menopausal breast cancer incidence, while the Chinese group has the highest incidence in

the pre-menopausal age bracket. The novel observations have not been described previously, and these ethnic differences in patterns have not been described in other populations previously.

Further analysis of the ethnic differences by plotting the birth cohort incidence rates of breast cancer supported and strengthened our hypothesis and observation that there was a real increase in the Chinese group, seen increasingly in each birth cohort, that was not present in the other two ethnic groups (as yet). The smaller numbers in the Malay group may have made a trend less easy to identify, but the recent large increase in the most recent (youngest) age groups was clear, but this was not seen in the other birth cohorts in the Malay group.

In our case-control study, we demonstrated that late age of marriage and having fewer children in Chinese women are two important risk factors of breast cancer. This is consistent with the results previous reported by Ng et al in Singapore [Ng, et al, 1997]. Compared to the Chinese group, Indian and Malay women tend to marry earlier, have children earlier and have more children. We have demonstrated this in our present case-control study (Section 4.3.3). From our primary results, Indian women have the lower incidence rate of breast cancer in the pre-menopause sub-group compared with Chinese women. This is consistent with the report of Gajalakshmi, et al [1991], who suggested that the protective effect of early full-term pregnancy is probably related to the prevention of tumor initiation.

In addition, our finding supports the idea that a long duration of lactation reduces breast cancer [Newcomb, et al, 1994]. We demonstrated that an increase in the period of breast-feeding has a protective effect against breast cancer in the Chinese group. These findings are similar to many other published studies [Pathak, et al, 1992; Kvale and Hench, 1988]. They also are consistent with the previous study in Singapore [Ng, et al, 1997]. One unexpected finding is that the percentage of non-breast-feeding in Chinese women is much higher than that of Malay and Indian (Section 4.3.3). This also could be one of the explanations why young Chinese women have a higher risk than other ethnicities.

We found that the experience of abortion increases the risk of breast cancer, especially in the women older than 30 years. Pike suggested that pregnancy precipitates an increase in hormone levels, but if this process is artificially interrupted (abortion), the hormone levels drop dramatically which renders the breast cells "vulnerable" to carcinogens. Abortion related variables were only examined in Chinese women because few Malay and Indian women in our subjects had this experience. This may be another reason for the higher incidence of breast cancer in Chinese women in Singapore.

Singapore is a multi-racial country with a high proportion of new immigrants. From the report of Cancer Incidence in Five Continents [Waterhouse et al, 1993], it was noted that there was a higher incidence of breast cancer in Indians (34 per 100,000) compared to others in their native counties (Chinese 31.6 per 100,000 and Malay 23.2 per 100,000). In Singapore, the varying lifestyles of the three ethnic groups are still retained to some extent, especially in the older post-menopausal group of women. This may be a possible explanation why Indian women had the highest incidence rate in the postmenopausal

women compared to others. In addition, in our case-control study (Section 4.3.4), we found that older Indian women have the highest percentage of higher body mass index compared with Malay and Chinese women. This could be consistent with the suggestion that weight gain in later life is associated positively with risk for breast carcinoma, regardless of body mass index in early adulthood [Chu SY, et al, 1991].

Many studies [Dewaard, et al, 1964; Topper, et al. 1980; Pike, et al, 1983; Russo, et al, 1995] have suggested that there are a variety of different mechanisms for the etiology of breast cancer. One of the theories propounds the view that "Breast tissue age" is an important factor of pre-menopausal breast cancer. This is closely related to the age of menarche, the age of first term of full pregnancy and the age of menopause. Coincidentally, Indian women have the earliest age of all 3 of the above items (Section 4.3.3). Maybe this is the reason that Indian women have a lower incidence than Chinese women in pre-menopausal breast cancer.

Malay women have a lower incidence but a greater annual increase of breast cancer compared with Chinese and Indian women. It should be noted that Malay women had the lowest incidence rate than others in their native countries [Waterhouse, et al, 1993]. Therefore this lower incidence could be related to their different lifestyles, and a host of other socio-cultural differences between the races [YIP & Ng, 1996]. In our case-control study, we found that the median age at presentation of breast cancer in Malays was younger than Chinese and Indian women, and more than half of the patients were premenopausal, in contrast to Indian women, whose two-third were postmenopausal. Younger Malay women may have experienced greater changes with regard to risk factors

[YIP & Ng, 1996] for breast cancer compared with their older generation, which may have led to the average annual change increase. A finding in our case-control study was that OC (Oral Contraceptive) use increased the risk in Malay women (Section 4.3.3) and this risk was even higher than in their Chinese counterparts. Many other studies have shown that Oral contraceptive use increases the risk of breast cancer [Spicer, et al 1994; Palmer, et al 1995; Brinton, et al 1997]. However, since many lifestyle related factors, such as diet, working stress, exercise were not investigated in our case-control study, we cannot give further suggestions why Malay women had the greatest annual increase in this study.

In our case-control study, the results indicate that Chinese, Malay and Indian women shared a common subset of some risk factors for breast cancer but that other subsets of factors related to breast cancer were distinguishable between the three ethnic groups. Cycle length of menses period, menopause status, age at first marriage, number of full term pregnancies, age at first pregnancy and oral contraceptive use, were in the subset of distinguishable factors related to breast cancer risk among Chinese, Malay and Indian women in Singapore. These may help to provide an explanation for different patterns for incidence rates of breast cancer. Although our study has focused on hormone related factors, the effect of hormonal factors may be mediated by dietary intake [Maclure M, et al, 1991], genetic factors and other factors [Fries H, et al 1974]. The prevalence of these factors also varies by ethnicity [Maclure M, et al, 1991].

5.2 Validity of the results

5.2.1 Validity and reliability data of the primary study

The findings observed in our primary study are likely to be reliable and real due to the following reasons.

The information notified to the Singapore Cancer Registry shows a high level of reliability. Up to 89.7% of total breast cancer cases for females were confirmed by histopathological reports in the period from 1968 to 1997, the remainder being diagnosed by clinical, radiological or other examinations. Our validation study demonstrated the accuracy of demographic data obtained. Moreover, the definitions of disease were consistent throughout this study and coding practice has not been changed over time.

We believe that referral patterns are not a problem here since the study was population-based and all diagnosed cases were included between 1968 and 1997. The registry receives notifications from all medical practitioners and quality control checks through all histopathological reports and hospital discharge summaries (Chia et al, 1996 & 2000). In this way, close to complete case ascertainment is ensured, as Singapore is a small island country with good communication links.

5.2.2 Quality of the data in the case-control study

The strong point of the present case-control study is the inclusion of only histopathologically confirmed cases of breast cancer. We excluded non-primary sites that manifested as breast cancer, through a careful review of the medical records of all cases.

The study population had the added advantage of being relatively homogeneous in that all the patients and controls had lived in Singapore for more than five years. This allowed us to examine the exposure of interest without the confounding effects of factors associated with these attributes. As the denominators of Malay and Indian populations are lower in Singapore [Lau, 1992], we faced difficulty in interpreting the risk estimates among Malay and Indian because of the much smaller numbers involved. Comparatively, the large number of Chinese in our study population allowed us to estimate fairly precisely the odds ratios for this group.

All interviews were conducted in-person by the same interviewer. Thus the uncertainty associated with proxy interviews was avoided. The potential for interviewer bias in which knowledge of the patient's disease status may influence the intensity of the search for the putative cause, exists in all case-control studies. In order to avoid that, we tried to clearly define the questionnaire items before patients were interviewed. On the other hand, differential recall of exposure between cases and controls should also be considered; this is usually more problematic when community controls are used [Henneken and Buring, 1996], which is not the case in the present study. Further, in introducing the study to eligible participants, the general term "women's health" was used and not "breast cancer".

We also gathered information from cases and controls concurrently. Controls were matched to cases on age at admission $\pm$5 years. We chose to use hospital, rather than population controls, the reasons for this choice being primarily concern that participation rate in the community would not be high. Whereas the use of hospital controls is considered by some to be less desirable than population controls, in a disease like breast cancer, this is likely to be less important than diseases which are more chronic and which receive more community care.

From our arguments above, we therefore believe that the quality of the data in the present case-control study is adequate to allow us to consider closely the findings and their implications.

5.3 Conclusions

We conclude that

1. In Singapore, the three main ethnic groups, Chinese, Malay and Indian have all had strikingly significant increases in incidence of breast cancer over the past 3 decades.
2. The changes in incidence rates of breast cancer in three ethnic groups have different patterns.
    - The greatest incidence rate is in the Chinese group while the highest annual increase is in the Malay group.

- The Indian group has the highest post-menopausal breast cancer incidence, while Chinese group has the highest incidence in pre-menopausal breast cancer.

3. The case-control study indicated that certain identified hormonal related risk factors might differ by ethnic groups.
4. There are some limitations in our case-control study:
   - The variances of the relative risk estimates are greater for Malay and Indian women because of the smaller sample size.
   - Some other risk factors, such as dietary intake, immigration, genetic factors, which are indirectly related with hormonal factors, have not been studied.

Chapter 6 Introduction

Several studies have shown an association between body mass index and breast cancer (See Part I). As body mass index is an indirect measure of body fat, there are some suggestions that the relation of breast cancer and body mass index is primarily because of body fat. One of the possible mechanisms may be via estrogen excess in breast tissue, due to direct peripheral androgen aromatization in fat tissue.

Recently, human leptin has been discovered and been described to be produced by adipose tissue. While the main function of the leptin is thought to be involved in the control of body fat by the regulation of energy homeostasis, other roles have been investigated also. For example, it is now thought that leptin might play an important role in reproduction and during gestation [Butte, et al, 1997].

Leptin is produced in adipose tissue [Zhang, et al, 1994] and this protein is a 167 amino acid protein transcribed from the obesity gene. Leptin achieves most of its metabolic effects by interacting with its specific receptor [Campfield, et al, 1995]. The leptin receptor is a member of the class I cytokine receptor family, a family that also includes the interferon receptor, growth hormone receptor and interleukin-2 receptor. The leptin receptor has been identified and described in many human tissues, such as ovarian cell, hepatic cell, pancreatic ß-cell and others. Evidence also showed that the leptin receptor and its mRNA are present in ovarian tissue of rodent and human. Whereas the leptin

receptor has been described in human breast tissue, it has not been investigated much more.

Generally, leptin reflects the amount of energy stores that regulates energy balance and is associated with circulating levels of reproductive hormones. Breast cancer has also been associated with obesity, reproductive hormones and circulating. To investigate whether leptin is related with proliferation of breast cancer, the effect of exogenous leptin on the breast cancer cell line, MCF-7 was studied in vitro. Furthermore, cDNA microarray was used to investigate the effect of leptin on breast cancer.

Chapter 7 Literature review

7.1 Leptin

In 1994, the obesity gene was cloned and its encoded 167 amino acid (novel leptin) was identified. [Zhang, et al, 1994]. The mature polypeptide is a 16-kDa adipocyte secreted molecule found in the blood of multiple mammalian species including mice and humans [Maffei, et al, 1995]. Although the primary amino acid sequence of leptin appeared unrelated to any known proteins, threading analysis performed to gauge tertiary structure suggested leptin would fold into a helical cytokine-like structure similar to interleukin-2 (IL-2) and growth hormone (GH)[Madej, et al, 1995].

Leptin expression has been shown to respond to a variety of stimuli including glucocorticoids, cytokines and insulin [Saladin, et al, 1996; Grunfeld, et al, 1996; Slieker, et al, 1996]. More importantly, the steady-state levels of leptin mRNA and its protein product are elevated in a variety of animal obesity models and appear to correlate with the percentage of body fat [Maffei, et al, 1995; Frederich, et al, 1995; Frederich, et al, 1995]. These findings have fostered the idea that leptin serves as an “adipostat”, informing the body of the available energy stored in the adipose tissue.

Leptin mRNA and protein is also regulated in humans by both changes in percentage body fat as well as acute changes in food intake. Expression is upregulated in individuals with increased body fat and is down-regulated during body weight reduction [Considine,

et al, 1996; Hamilton, et al, 1995; Lonnqvist, et al, 1995].The administration of recombinant leptin to rodents results in food intake reduction and weight loss[Campfield, et al, 1995; Halaas, et al, 1995; Pelleymounter, et al, 1995; Stephens, et al, 1995]. A recessive mutation in the ob gene caused severe hereditary obesity in the ob/ob mouse, while the injection of exogenous leptin protein reversed the obesity [Sinha, et al, 1997].

7.2 Biologic effects of leptin

Initial models of leptin action included ob/ob mice, which are leptin deficient, and db/db mice, which are leptin insensitive due to a receptor defect. Peripheral or central administration of leptin reduced the body weight, adiposity and food intake of ob/ob mice but not of db/db mice [Campfield, et al, 1995; Pelleymounter, et al, 1995]. The behavioral effects after administration to the brain suggests that leptin can act directly on neuronal networks, which control food intake and energy balance [Campfield, et al, 1995; Cusin, et al, 1995; Hamann, et al, 1996]. It has been shown that leptin acts by both suppressing food intake and by stimulating energy expenditure, including thermogenesis [Campfield, et al, 1995; Halaas, et al, 1995].

Leptin suppresses NPY (neuropeptide Y system) expression and secretion in the arcuate nucleus. NPY is a strong stimulator of food intake and is involved in the regulation of various pituitary hormones. Leptin decreases food intake through inhibition of NPY mRNA expression, which in turn can cause a decrease in insulin levels. Low insulin

levels on the other hand cause a down regulation of leptin [Tomaszuk, et al, 1996; Stephens, et al, 1995; Spitzweg and Heufelder, 1997].

In addition to its effect on energy balance, leptin is also important for normal progression of pregnancy and for female reproduction and sexual development. Leptin corrects the infertility in the ob/ob mouse possibly by directly influencing the GnRH-LH/FSH axis [Chehab, et al, 1996]. It has been postulated that leptin stimulates the production of reproductive hormones. Subjects with a low body fat mass have disrupted reproductive systems, as do people who are starving. Women stop ovulating and testosterone levels decline in men [Kopp, et al, 1997]. Leptin may well be one of hormonal factors which signal to the brain at what time the body is ready for sexual maturation and reproduction. [Lahlou, et al, 1997].

Leptin levels in maternal serum are high throughout pregnancy [Butte, et al, 1997]. During late pregnancy and at birth when maternal fat stores are developed and leptin levels are high, leptin could be an important signal to the brain, signaling the status of satiety and expansion of fat stores. Also, high levels of leptin might lead to uncoupling of eating behavior, to the filling up of fat stores and to a relative unresponsiveness of leptin receptors during late gestation. Teleologically, it would make sense to prepare additional energy stores for the stress of birth and allowing for adequate lactation afterwards [Gluckman, et al, 1996].

7.3 Leptin receptor and signal transduction

Leptin acts through interactions with specific receptors [Campfield, et al, 1995]. The leptin receptor (Ob-R) is a large single membrane spanning protein and belongs to the cytokine class I receptor family [Tartaglia, et al, 1995]. Class I cytokine receptors contain highly conserved Box 1 and Box 2 motifs proximal to the transmembrane domain [Murakami, et al, 1991]. Box motifs mediate the association of Janus-activated kinase (JAK) family members with cytokine receptors, allowing the cytokine receptor to initiate an intracellular signaling cascade. While membrane-bound leptin receptor isoforms contain a Box 1 motif, only the long form of the receptor (OB-Rb) isoform contains an additional Box 2 motif. The OB-Rb isoform is also the only leptin receptor that contains intracellular tyrosine residues, which upon phosphorylation, allows interaction with specific signaling proteins [Tartaglia, et al, 1995and Tartaglia, et al, 1997]. Whereas signaling of the leptin receptor is similar to interleukin 6 (IL-6)-type cytokine receptor signaling, activation of OB-Rb is independent of the signal transducing subunit gp130, which is necessary for activation of the IL-6 receptor [Tartaglia, et al, 1995; Baumann, et al, 1996; White, et al, 1997]. Instead, leptin receptor signaling appears to be initiated by ligand-induced receptor homo-dimerization [White, et al, 1997].

A considerable amount of work has been performed to elucidate the intracellular signaling pathway regulated by leptin. This work has implicated JAK family members, signal transducers and activators of transcription (STAT), as well as mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathways in leptin receptor-mediated signal transduction [Baumann, et al, 1996; Bjørbæk, et al, 1997;

Banks, et al, 2000; Rosenblum, et al, 1996; Yamashita, et al, 1998; Ghilardi, et al, 1996]. (See Figure 7.3.1) Transfection studies have demonstrated that ligand binding to OB-Rb results in activation of the JAK/STAT signaling pathway, whereas cells transfected with OB-Ra did not display leptin-induced JAK/STAT activation [Ghilardi and Skoda, 1997]. Furthermore, administration of leptin to female ob/ob and wild type mice activates hypothalamic STAT3. In contrast, db/db mice, which have a truncated OB-Rb, fail to exhibit an increase in STAT3 activation, implicating the OB-Rb isoform in mediating the biologic effects of leptin, in vivo [Vaisse, et al, 1998]. The inability of leptin to induce STAT3 activation in db/db mice appears to be due to the fact that db/db mice lack a YXXQ motif in the cytoplasmic tail of the OB-Rb, which represents a consensus STAT3 binding motif [Tartaglia, et al, 1995]. Finally, recent research by several groups has implicated the nuclear transcription factors c-fos, c-jun and egr-1 as being regulated by leptin and potentially participating in leptin signaling [Murakami, et, al, 1997; Uotani, et al, 1994]. Further work is needed before a precise understanding of the contribution of these factors to leptin signaling can be reached. Nevertheless, as a whole, the above findings suggest STAT3 regulation of target genes plays an important mediator role in leptin signaling, which appears to be critical to the ability of leptin to regulate food intake and energy metabolism in vivo.

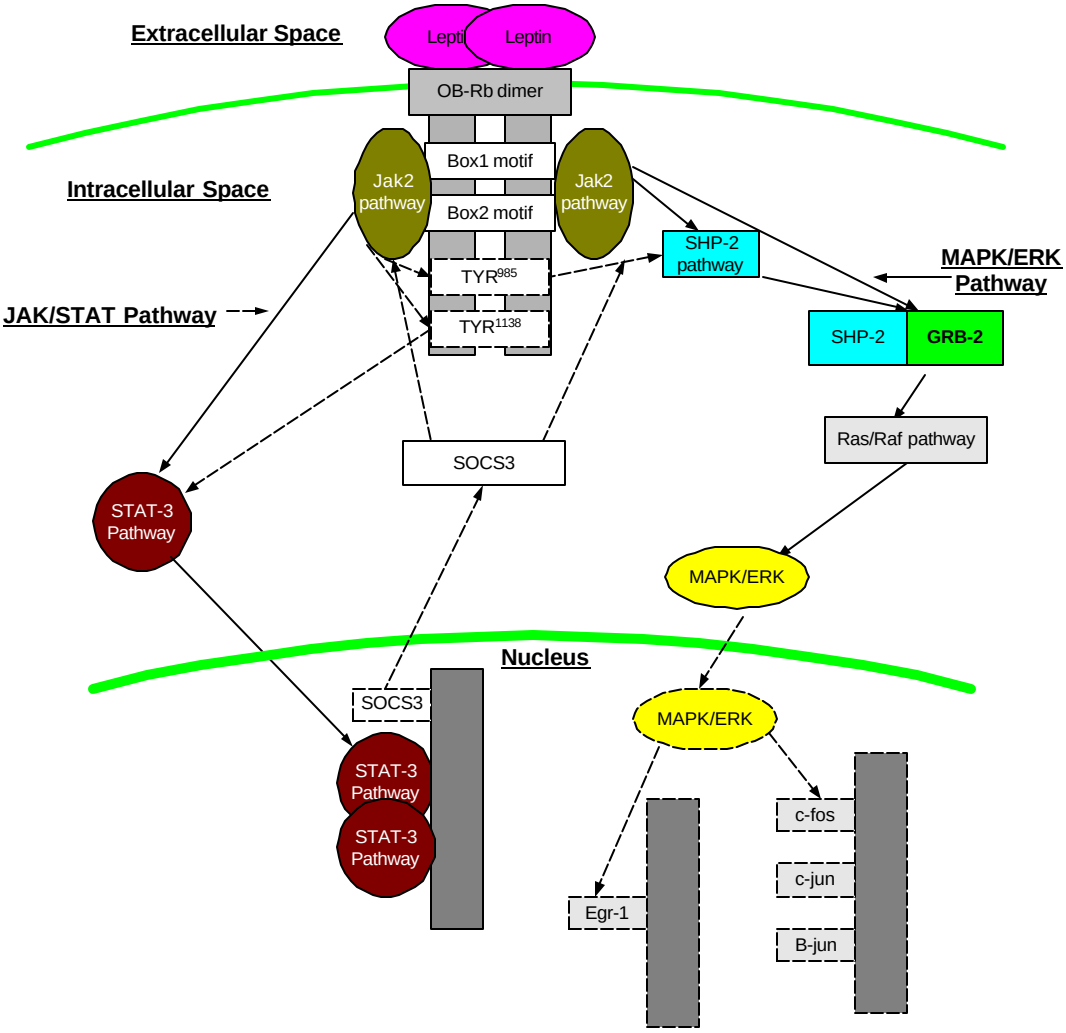
While the pathways responsible for mediating the actions of leptin have received considerable attention, less is known about the mechanisms that limit leptin signaling. Recent studies on this issue have demonstrated that STAT3 induces gene transcription of the suppressor of cytokine signaling-3 (SOCS-3) [Bjø rbæk, et al, 1998]. The SOCS family of proteins consists of eight members, each of which contains a Src-homology

(SH2) domain and a C-terminal SOCS box [Starr, et al, 1997]. SOCS proteins are induced by a wide variety of cytokines and function to inhibit cytokine-mediated signal transduction, thus serving a negative feedback role. Recent work demonstrated that overexpression of SOCS-3 results in the inhibition of leptin-induced tyrosine phosphorylation of JAK2, thus preventing the activation of the JAK/STAT pathway following the addition of leptin [Bjørbæk, et al, 1999]. Subsequent studies determined SOCS-3 also inhibits leptin signaling by binding to phosphorylated Tyr-985 on the leptin receptor, thereby blocking the interaction of this residue with SH2-containing phosphatase (SHP-2), a tyrosine phosphatase [Bjørbæk, et al, 2000; Li and Friedman, 1999]. SHP-2 is ubiquitously expressed and generally acts to positively regulate cytokine signaling. Following cytokine receptor stimulation, SHP-2 becomes tyrosine phosphorylated and acts as an adaptor molecule to recruit Grb2 and Sos, members of the Ras/MAPK/ERK signaling pathway. Using a dominant negative SHP-2 construct, Bjø rbæk et al. demonstrated SHP-2 is essential for leptin-induced MAPK phosphoyrlation by OB-Rb [Bjø rbæk, et al, 2000]. In contrast, a recent study reported that SHP-2 acts as a negative regulator of OB-Rb signaling by inhibiting STAT3 mediated transcription [Carpenter, et al, 1998]. Thus, the precise role of SHP-2 in leptin signaling has not been completely resolved.

Nevertheless, based on the studies described above, it can be concluded that leptin signaling involves mediator roles for the JAK/STAT and MAPK/ERK pathways, and that SOCS3 may serve to limit/restrain leptin action. These intracellular signaling pathways suggest some overlap with those of other growth factors, and of Growth Hormone in

particular [Kaulsay, et al, 2000]. The presence of these overlapping signaling pathways may indicate a possible involvement of leptin in breast cancer.

Figure 7.3.1 Proposed signaling model of the leptin receptor OB-Rb isoform



Positive regulation

Negative regulation

Leptin receptor signaling is initiated by the binding of leptin to the extracellular domain of the OB-Rb dimer. Jak 2 binds to Box 1 and Box 2 motifs on the receptor, leading to Jak 2 transphosphorylation and activation. Activated Jak 2 subsequently phosphorylates Tyr^{985} and Tyr^{1138} of the leptin receptor. Phosphorylated Tyr^{985} and Tyr^{1138} immediately bind SHP-2 and STAT-3, respectively, which are then phosphorylated by Jak 2. Tyrosine-phosphorylated SHP-2 binds GRB-2, resulting in the activation of the Ras/Raf MAPK/ERK pathway. GRB-2 may potentially also be activated directly by Jak 2. Activated MAP/ERK translocates to the nucleus, where it induces transcription of immediate early response genes such as c-fos, c-jun, and B-jun, as well as egr-1. Tyrosine-phosphorylated STAT-3 dimerizes and translocates to the nucleus, where it induces transcription of STAT-3-responsive genes, including SOCS3. After socs3 mRNA has been translated and translocated to the cytoplasm, SOCS3 binds to phosphorylated Tyr^{985} and blocks the SHP-2/MAPK/ERK pathway and may also bind Jak 2 directly to negatively regulate leptin receptor signaling.
Sourc:Brann, et al, 2002

Chapter 8 Materials and Methods

MCF-7 cells were cultured at 37°C in 5% CO_2 in RPMI supplemented with 10% heat-inactivated FBS, 100U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine.

8.1 Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

Total cellular RNA was extracted from MCF-7 cells by the RNeasy Mini Kit (QIAGEN). According to the protocol (QIAGEN OneStep PCR Kit), 5x OneStep RT-PCR buffer, DNTP mix, 5x Q-solution and RT-PCR Enzyme Mix were prepared for a master mix. cDNA was synthesized from 1µ g of total RNA. For amplification of the cDNA products, human leptin receptor specific primers (Primer 1:sense primer, 5- tgttgtgaatgtcttgtgcc-3 and anti sense primer 5-cattagacccaacacttgtc-3 or Primer 2:sense primer, 5-tgttgtgaatgtcttgtgcc-3 and anti sense primer 5-tggaaatggtaccaatggtg –3) were added in the individual PCR tubes. PCR was performed on the Gene Amp PCR system 9600 .The details of thermal cycles are following: Reverse transcription 50°C for 30min, Initial PCR activation step 95°C for 15min, 3-step cycling for 35 circles and Final extension at 72°C for 10min. Each cycle was carried out 30s denaturation step at 94°C, 30s annealing step at 52°C and extension at 70°C .PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide.

8.2 Western blot analysis

MCF-7 cells were washed once in ice-cold PBS and scraped into 1 ml of 1x lysis buffer [2x lysis buffer: 2% Triton X-100, 20mM Tris-HCl, pH 7.4, 300 mM NaCl, 2mM EDTA, 2mM EGTA, 0.4mM Na_3VO_4, 1% Nonidet P-40, 0.1% phenylmethylsulfonyl fluoride (PMSF)]. Lysate was incubated on ice for 15 min and then centrifuged for 20 min at 4°C and 12,500 x g, and the supernatant was collected. Protein concentration was determined in triplicate by the Bro-Rad protein assay using bovine serum albumin (BSA) as the standard. Protein was resuspended in 2x SDS-sample buffer (50mM Tris, pH 6.8, 2% SDS, 2% ß-mercaptoethanol, and bromophenol blue), boiled for 10 min, and centrifuged at 14,000 x g for 2 min. Protein was separated by 7% SDS-PAGE [1.5M Tris-HCl, pH 8.8, 20% (w/v) SDS, acrylamide/bisacrylamide (30%/0.8% w/v), 10% (w/v) ammonium persulfate, 0.05%TEMED] in 1xLaemlli running buffer [5x Laemlli running buffer: 120mM Tris (hydroxymethyl) methylamine, 960 mM glycine, 17mM sodium dodecyl sulfate (SDS)], transferred to a nitrocellulose membrane using a semidry apparatus in Laemlli buffer [23mM Tris (hydroxymethyl) methylamine, 19mM glycine, 0.64 mM SDS] containing 10% methanol. Subsequently, membrane was blocked with 5% skim milk in PBS with 0.1% Tween 20 (PBST) for 1h at room temperature and then washed twice for 5 min each in PBST. Immunoblotting of the membrane with monoclonal antibody against leptin receptor (1:1000) was performed for 1h at 22°C with 1 x PBST containing 1% skim milk powder. After six washes for 10 min each in PBST, the membrane was incubated with anti-rabbit HRP (1:10,000) (Amersham, Corp.) in PBST containing 1% skim milk powder for 1h at 22°C. The membrane was further washed six times for 10 min each in PBST, and revealed using the enhanced chemiluminescence (ECL) system.

8.3 Confocal Laser Scanning Microscopy for leptin receptor

MCF-7 cells were cultured on glass coverslips, fixed in ice-cold 4% paraformaldehyde (in 1 x PBS pH 7.4), washed in 1 x PBS, permeabilized for 10 min with 0.1% Triton X-100 (in 1 x PBS), blocked for 1 h at 22°C in 1 x PBS containing 2% BSA, and incubated with either a monoclonal antibody against Leptin receptor (dilution 1:150) or with Rabbit anti-mouse serum (dilution 1:150) in incubation buffer (1 x PBS, pH 7.4, 1% BSA). For detection, FITC (1:150) and phalloidin-TRITC (1:1000) labeled goat anti-rabbit diluted in incubation buffer (1% BSA/PBS, pH 7.4)) was used at 22°C. After 5 washes in PBS the coverslips were mounted and labeled cells visualized with a Carl Zeiss Axioplan microscope equipped with epifluorescence optics and a Bio-Rad, Inc., MRC1024 confocal optics system. Images were converted to the tagged information file format (TIFF) and processed with the Adobe Photoshop program.

8.4 Cell proliferation assay using 5-Bromo-2'-deoxyuridine (BrdU) staining

Mitogenesis was directly assayed by incorporation of 5-Bromo-2'-deoxyuridine (BrdU) during DNA synthesis (Sawa et al., 1999). MCF-7 cells were cultured on the glass coverslips to 25% confluence in six-well plates. Cells were washed twice with PBS before being serum deprived for 12 hours. Cells were treated in either serum-free RPMI medium or serum free medium supplemented with 100nM leptin or serum free medium supplemented with 10nM U0126 or serum medium supplemented with 10nM U0126 then treated with100nM leptin. 24 hours later, all cells were pulse-labeled with 20 mM BrdU (Sigma) for 30 min, washed twice with PBS, and fixed in cold 70% ethanol for 30

minutes. BrdU detection was performed by using the BrdU staining Kit (Zymed, South San Francisco, CA) according to the manufacturer's instructions, A total population of over 400 cells was analyzed in several arbitrarily chosen microscopic fields to determine the BrdU labeling index (percentage of cells synthesizing DNA).

8.5 Mitogen-activated protein (MAP) kinase activity in MCF-7 cells

MAP kinase activity was measured with the p44/42 MAP Kinase Assay kit (Cell Signaling Technology Catalog #9800), according to the manufactures recommended protocols.

8.5.1 Immunoprecipitation of proteins from cell extracts

MCF-7 cells were grown to 50% confluence in 10% serum-supplemented medium, incubated for 12 hours in serum-free medium and stimulated by indicated dose leptin for 15minutes (for Dose-response experiments) or by 100nM leptin for indicated time periods (for Time course experiments). Cells were washed with ice-cold PBS and lysed with 1ml of lysis buffer [20mM Tris (pH7.5), 150 mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1mM ß-Glycerolphosphate, 1mM Na_3VO_4, 1µg/ml Leupeptin, 1mM PMSF]. After micro-centrifugation for 20min at 4°C, the supernatant was subjected to immunoprecipitation using the Phospho- 44/42 MAPK (Thr202/Tyr204) monoclonal antibody with agarose beads. After overnight agitating at 4°C, micro-centrifuge for 30 seconds at 4°C, washing pellet twice with ice-cold lysis buffer, then washing with ice-cold kinase buffer [25mM Tris(pH 7.5), 5mM ß-Glycerolphosphate, 2mM DTT, 0.1 mM Na_3VO_4, 10mM $MgCl_2$], the pellets were

incubated for 30 min at 30°C with the kinase buffer containing 200µM ATP and 2 µg Elk-1 fusion protein. The reaction was stopped by the addition of 25µl 3x SDS sample buffer and then 30µl of each sample loaded on SDS-polyacreylamide gel electrophoresis (PAGE). Proteins were transferred to nitrocellulose membranes using a standard semi-dry electroblotting apparatus in Laemlli buffer containing 10% methanol.

8.5.2 Western Blot Analysis

Nitrocellulose membranes were blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline with 0.1% Tween 20 (PBST) for 1 hour at 22°C. Blots were then immunolabelled for 1 h at 22°C with rabbit phosphor-specific Elk1 (Ser383) antibody (1:1000). After 6 washes for 10 min each in PBST, membranes were incubated in horseradish peroxidase-conjugated anti-rabbit for 1 h at 22°C, which were diluted with the buffer [20mM Tris (pH7.5), 150 mM sodium chloride, 5% skim milk, 0.1% Tween 20]. Membranes were further washed six times for 10 min each in PBST before immunolabelling detected by ECL according to the manufacturer's instructions.

8.6 cDNA Array Hybridization Studies

8.6.1 Preparation of Total RNA

Total RNA was isolated from MCF-7 cell lines and MCF-7 cells treated with 200nM leptin separately, using the TRI REAGENT method (MOLECULAR research center, inc) according to manufacturer's instructions and resuspended in diethyl pyrocarbonate-treated water. Quantification and purity of the RNA was assessed by A260/A280

absorption, and RNA quality was assessed by agarose gel electrophoresis. RNA samples with ratios greater than 1.6 were stored at 70°C for further analysis.

8.6.2 Analysis of Differential Gene Expression by Use of cDNA Microarray

The Atlas cDNA Expression Arrays (Clontech Laboratories, Palo Alto, CA) containing 588 genes were used in these studies. Poly(A)+ RNA samples from the respective cell lines were isolated from total RNA using streptavidin magnetic beads. Following the manufacturer's instructions, Moloney murine leukemia virus reverse transcriptase was incubated with Poly(A)+ RNA in the presence of [a-32P]dATP for generation of radiolabeled cDNA probes. Before labeling for hybridization to the cDNA microarray, the radiolabeled cDNA probes were purified from the unincorporated nucleotides by gel filtration in chromatography-spin 200 columns. The product was then hybridized to the cDNA microarray overnight. After a series of high stringency washes (three 20-min washes in 2× saline/sodium citrate (SSC), 1% SDS followed by two 20-min washes in 0.1× SSC, 0.5% SDS), at 68 °C the membranes were exposed to x-ray film and subject to autoradiography. The relative levels of gene expression were quantified by densitometric scanning by the use of the GS-700 imaging densitometer from Bio-Rad according to the manufacturer's instructions. Genes were considered differentially expressed when they exhibited a 2-fold or greater increase or decrease in the presence of leptin (MCF-7 cells) compared with the absence of leptin (MCF-7 cells) in three independently performed experiments. The relative expression of housekeeping genes (ubiquitin, phospholipase A_2, glyceraldehyde-3-phosphate dehydrogenase, -actin, -tubulin, 23-kDa highly basic protein, ribosomal protein S9) did not differ by more than 10% between MCF-7 cells with or without leptin.

8.7 Statistics

All experiments were repeated at least three, usually five times. All numerical data are expressed as mean ±S.D. Data was analyzed using the two-tailed t test or analysis of variance.

Chapter 9 Results

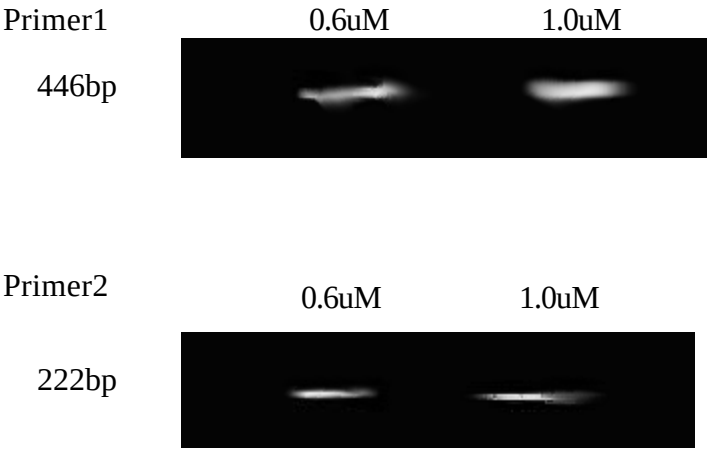
9.1 Expression of the leptin receptor in MCF-7 cell line

To demonstrate the presence of leptin receptor in MCF-7 cells, semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was used to display the presence of leptin receptor mRNA transcripts in MCF-7 cells. Using two different primer sets and different primer doses, amplified fragments of the predicted size (446bp and 222bp) appropriate for the leptin receptor mRNA fragment were detected in total RNA extracts from MCF-7 cells. (Figure 9.1.1)

Leptin receptor protein expression was also shown by Western blot analysis. OB-Rb protein was detected at 230 kDa by using monoclonal antibody against leptin receptor (Figure 9.1.2). Leptin receptor protein localization within the MCF-7 cell was also demonstrated by cofocal Laser scanning microscopy (Figure 9.1.3) with the use of a monoclonal rabbit leptin receptor antibody. For control purpose, another treatment was performed with MCF-7 cells by using rabbit anti-mouse serum for primary antibody. Both of them were detected with FITC and phalloidin-TRITC labeled goat anti-rabbit (second antibody). Leptin receptor immunoreactivity was detected in MCF-7 cells (Figure 9.1.3c). No immunofluorescence was detected if antibody against leptin receptor was substituted with a monoclonal antibody directed against a species-specific epitope on the rabbit leptin receptor (Figure 9.1.3a). As positive control, filamentous actin within both of the cells was visualized with TRITC-labeled phalloidin (Figure 9.1.3b & Figure 9.1.3 d).

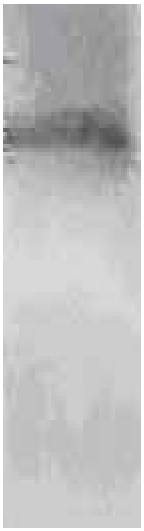
Thus, we provide clear evidence for both leptin receptor mRNA and protein expression in the human breast carcinoma cell line, MCF-7, in vitro.

Figure 9.1.1 Expression of the human leptin receptors mRNA in MCF-7 cell line.



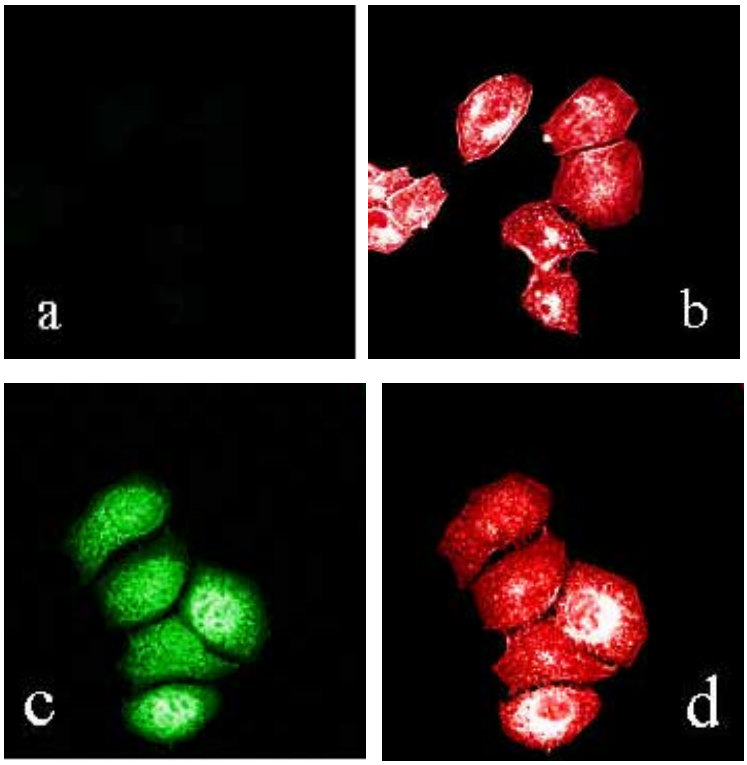
Reverse transcriptase-polymerase chain reaction demonstration of leptin receptor gene expression in MCF-7 cells. RT-PCR was performed as described under Materials and Methods to yield a product of 446 bp and 222 bp. Agarose gel electrophoresis of the amplified fragments from MCF-7 visualized with ethidium bromide. [mRNAs encoding long Ob-Rb]

Figure 9.1.2 Western blot analysis of the human leptin receptor protein expression in MCF-7 cell line.



Western blot analyses were performed as described under Materials and Methods. OB-Rb protein was probed by corresponding antibody and visualized by enhanced chemiluminescence. A single band for OB-Rb was detected.

Figure 9.1.3 Leptin receptor demonstrated in MCF-7 cells by cofocal laser scanning



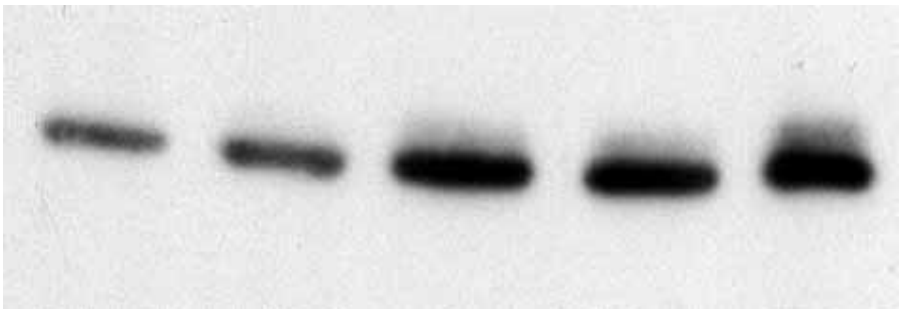
Leptin receptor was detected with the monoclonal antibody against Leptin receptor and confocal laser scanning microscopy. a, Lack of immunoreactivity in MCF-7 cells (detected with FITC-labeled second antibody); b, Positive filamentous actin control (visualized with TRITC-labeled phalloidin); c, Anti-leptin receptor immunoreactivity in MCF-7 cells (detected with FITC-labeled second antibody); d, Positive filamentous actin control (visualized with TRITC-labeled phalloidin).

9.2 Activation of MAPKinases pathway by leptin in MCF-7 cells

Previous studies have shown that leptin induced cell proliferation acts via the activation of the MAPKs pathway [Takahashi et al., 1997, Tanabe et al., 1997 and Tartaglia et al., 1995]. We measured MAPK phosphorylation after leptin stimulation in the MCF-7 cell line. As shown in Figure 9.2.1a, leptin stimulates phosphorylation of Elk-1, which is the p44/42 MAP kinase dependent transcription factor. We demonstrated that leptin activated MAPK pathway on MCF-7 in a dose-dependent manner. As shown in Figure 9.2.1b, we used concentrations of leptin 0, 1, 5, 50 and 100nM at a time duration of 15min. It was demonstrated that leptin stimulates phosphorylation Elk-1 with a maximal activation at 100nM.

We also used an optimal concentration of leptin (100 ng/ml), during various times of stimulation (0, 2, 5, 15, 30, 60 min). In the time-dependent manner, a maximal activation was detected at 15 min time point; at longer time duration, this activation diminishes.

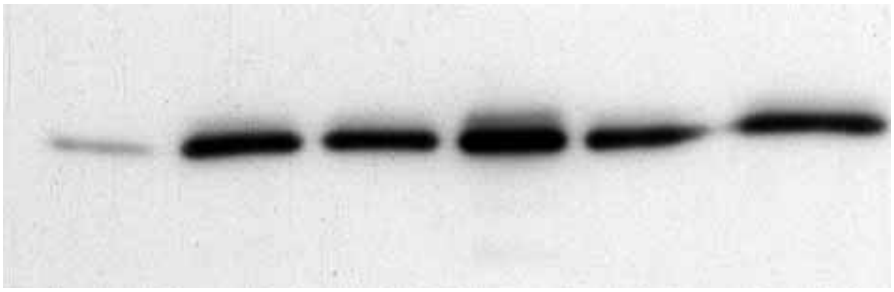
Figure 9.2.1 Dose and Time dependence of the leptin-stimulated phosphorylation of Elk-1 in MCF-7 cells.



Phospho-Elk-1

0 1 5 50 100

DOSE DEPENDENCE [nM] Figure9.2.1a



Phospho-Elk-1

0 2 5 15 30 60

TIME DEPENDENCE [MINUTES] Figure 9.2.1b

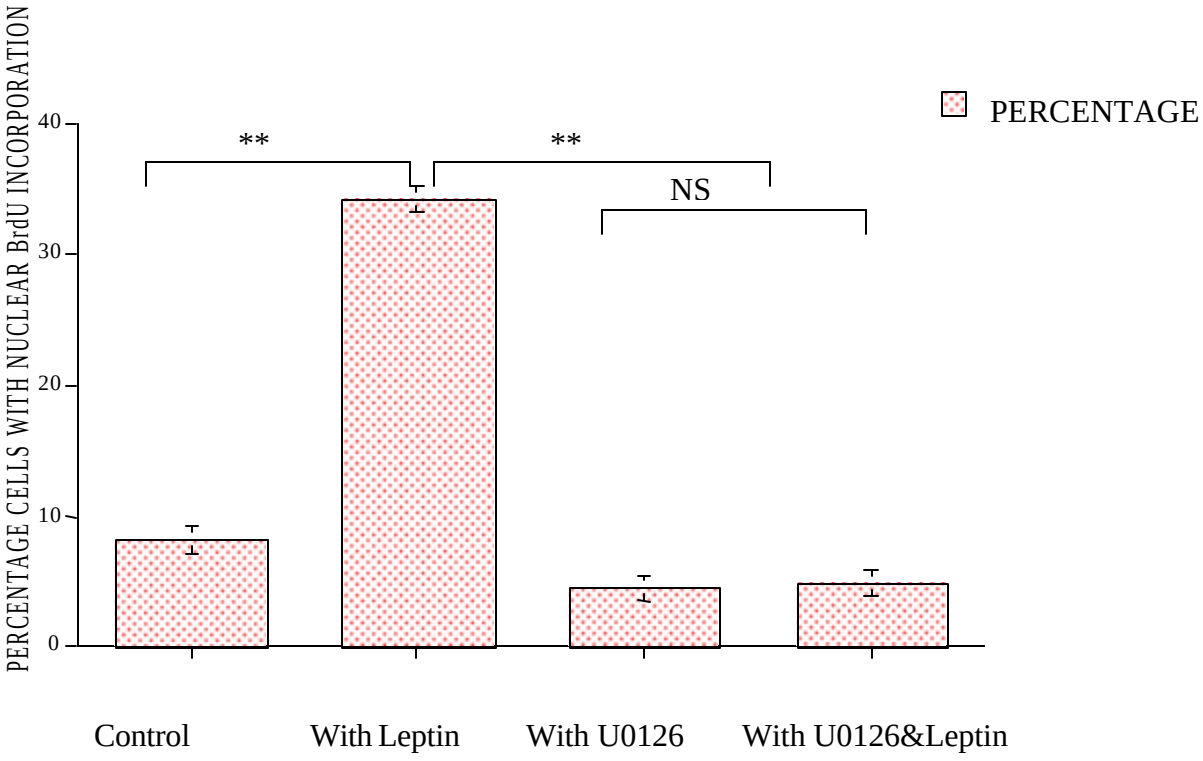
For dose dependence experiments, MCF-7 cells were stimulated with the indicated concentration of leptin for 15 min. For time course experiments, MCF-7 cells were stimulated with 100nM leptin for the indicated times. The data presented are representative of at least three separate experiments.

9.3 Leptin effect on cell proliferation in breast cancer cell line (MCF-7)

To study the functional role of the leptin receptors on human breast cancer, we evaluated the effects of leptin on MCF-7 cell line proliferation by BrdU incorporation assay. We demonstrated that leptin induces proliferation of the human breast cancer cell line MCF-7 in a dose-dependent manner with a maximal activation at 100 nM previously. The BrdU incorporation in MCF-7 cells was significantly increased with 100nM leptin (See Figure 9.3.1 & Figure 9.3.2)

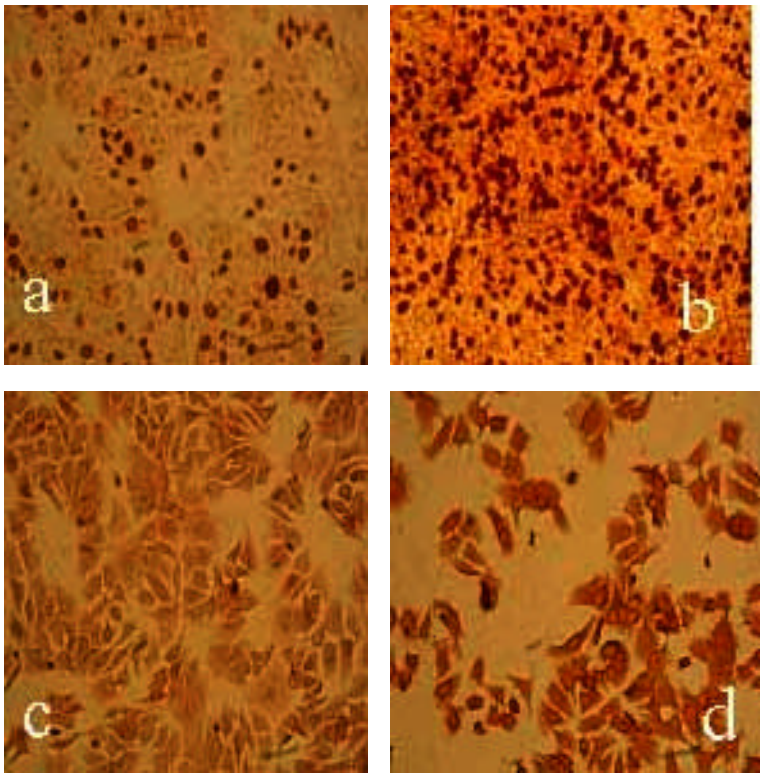
In order to determine if the MAPKs pathways activation is truly involved in leptin-induced proliferation of MCF-7 human breast cancer cell line, we studied the effect of the specific MAPK-inhibitor U0126 on the growth of MCF-7 induced by leptin. MCF-7 cell proliferation was inhibited when U0126 specifically inhibited the activation of the MAPkinase pathway by (See Figure 9.3.2c and Figure 9.3.2d). Thus, we demonstrated that this specific inhibitor, at a concentration of 10 nM, totally inhibits the leptin-induced cell proliferation.

Figure 9.3.1 Leptin-induced proliferation in p44/42 MAP kinase pathway in MCF-7 cells and 5'-bromo-2'-deoxyuridine (BrdU) corporation.



Cell proliferation assays were performed as described in Material and Methods. Results represent means ± SD of triplicate determinations. Results presented are representative of at least three independent experiments. **, P<0.01; NS: No Significance.

Figure 9.3.2 Leptin-induced proliferation in p44/42 MAP kinase pathway in MCF-7 cells and 5'-bromo-2'-deoxyuridine (BrdU) corporation.



Cell proliferation was estimated by the BrdU incorporation assay. Microscopy was performed as described in Material and Methods. a, MCF-7 cells in serum-free medium; b, MCF-7 cells stimulated with 100nM leptin; c, MCF-7 cells treated with 10nM U0126; d, MCF-7 cells treated with 10nM U0126 then stimulated with 100nM leptin. The results were shown from three independent experiments.

9.4 Microarray analysis

To identify genes regulated by human recombinant leptin in MCF-7 cells line, we screened a high-density cDNA array with labeled cDNA derived from either serum-free MCF-7 cells or stimulated with 200nM leptin for 6 hours MCF-7 cells. Of 588 screened genes, 23 exhibited a 2-fold or greater increase in their expression in the presence of human recombinant leptin (range 2~7.3 fold) compared with the absence of leptin (See Figure9.4.1a and Table 9.4.1). Most of the up-regulated genes were to be found among those grouped as oncogenes/tumor suppressors/ cell cycle control proteins; in particular, the dramatic effects of leptin up-regulations expression were on Cyclin-dependent kinase (CDK1), Mitogen-activated protein kinase 3 (MAP kinase 3; MAPK 3; PRKM3); Mitogen-activated protein kinase 3; Type ll cytoskeletal 2 epidermal keratin. Other up-regulated genes of particular interest include DNA topoisomerase l (TOP1).

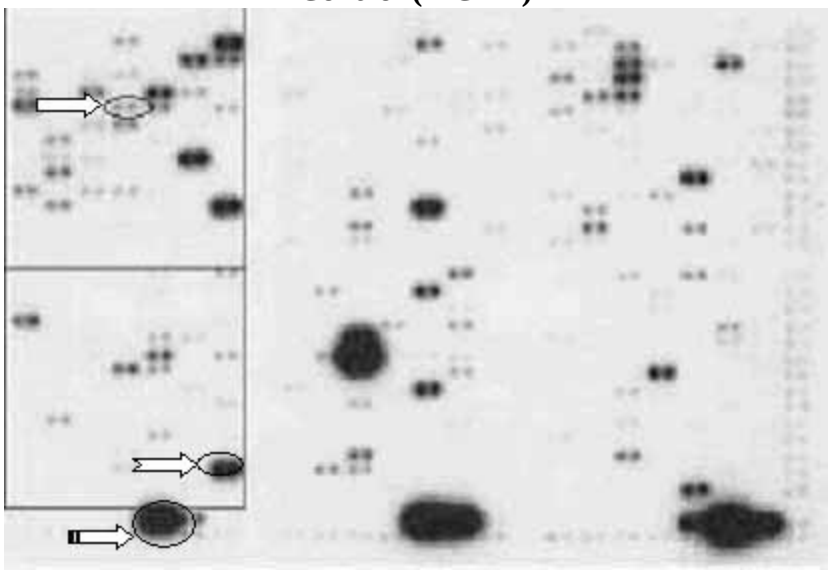
17 genes exhibited a 2-fold or greater decrease in their expression in the presence of human recombinant leptin (range 2~5.6 fold) compared with the absence of leptin treatment (Figure9.4.1 and Table 9.4.2). Most of the down-regulated genes were to be found among DNA binding/ cell cycle control proteins / transcription factors group. The effects of leptin down-regulations expression were on purine-rich single-stranded DNA-binding protein alpha (PUR-alpha), cyclin-dependent kinase 4 inhibitor (CDK4I), Interleukin 10 (IL10) and others.

The relative expression of housekeeping genes (ubiquitin, phospholipase A_2, glyceraldehyde-3-phosphate dehydrogenase, -actin, -tubulin, 23-kDa highly basic protein, ribosomal protein S9) did not differ by more than 10% between serum-free MCF-7 cells and leptin treated MCF-7 cells.

Figure 9.4.1 Effect of human recombinant leptin on relative levels of gene expression in MCF-7 cells

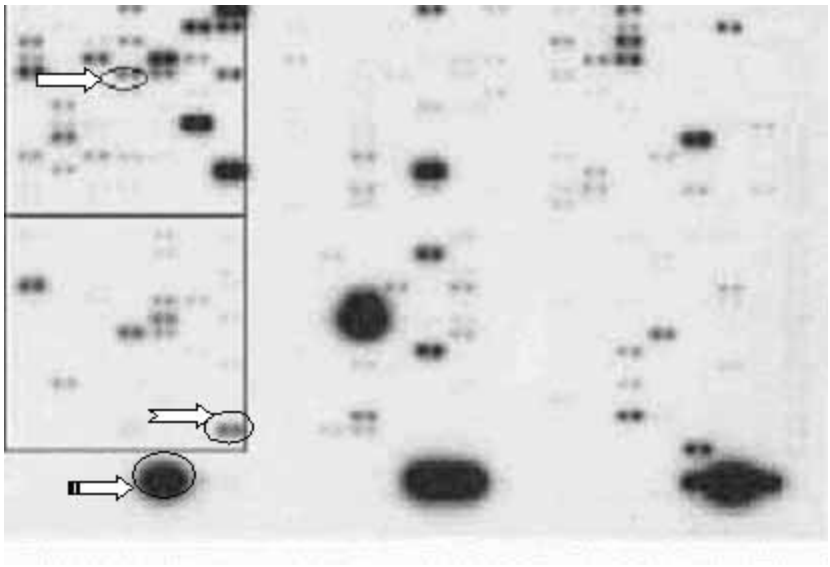
Control (MCF-7)

Figure9.4.1a



With Leptin (MCF-7)

Figure9.4.1b



⇨ Up-regulated gene; ⇨ Down-regulated gene; ⇨ Housekeeping gene

cDNA microarray analysis of the relative levels of gene expression in MCF-7 cells. Fig 9.4.1a, cultured in serum-free medium; Fig 9.4.1b, MCF-7 cells was serum-free 16 hours then treated with 200nM leptin for 6 hours. 32P-Labeled cDNA probes generated from ploy (A)+ RNA isolated from MCF-7 cells were hybridized to a cDNA microarray containing 588 known human genes. The left upper box and left lower box encase those genes grouped as oncogenes/tumor suppressors/ cell cycle control proteins and DNA binding/ cell cycle control proteins / transcription factors, respectively. The results were shown from three independent experiments. The position of MAPK3 cDNA is indicated by ellipse and the relative expression level of cDNA is up-regulated. The position of vascular endothelial growth factor receptor 1 is also indicated by ellipse and the relative expression level of cDNA is down-regulated.

Table 9.4.1 Identification by cDNA array of genes positively regulated by the human recombinant leptin in MCF-7 cells.

Genes were considered positively regulated when they exhibited a 2-fold or greater increase in the presence of leptin compared to the absence of leptin. Fold regulation is calculated as the ratio of the level of gene expression in treated with leptin MCF-7 cells to that without leptin treated MCF-7 cells.

Genebank Accession	Fold stimulation	Gene/Protein Name
X05360	7.3	Cell division control protein 2 homolog(CDC2); P34 protein kinase; Cyclin-dependent kinase (CDK1)
M14505	2.00	cell division protein kinase 4; Cyclin-dependent kinase 4(CDK4); PSK-J3
L29222	2.58	CDC-like Kinase1 (CLK1)
L29220	3.60	CDC-like Kinase3 (CLK3)
L25676	2.06	Cell division protein kinase 9(CDK9); serine/threonine protein kinase PITALRE
U00001	2.85	CDC27HS protein
L22005	2.24	Ubiquitin-conjugating enzyme E2 32-kDa complementing protein; ubiquitin-protein ligase; ubiquitin carrier protein, CDC34
U63131	2.70	CDC37 homolog
X60188	2.31	Mitogen-activated protein kinase 3 (MAP kinase 3; MAPK 3; PRKM3); extracelluar signal-regulated kinase1 (ERK1); ERT2
L31951	2.2	Mitogen-activated protein kinase 9 (MAPK 9; MAPk 9: PRKM9; c-jun N-terminal kinase 2 (JNK2)
M29039	2.0	Jun-B
M99061; S43636	4.38	Type ll cytoskeletal 2 epidermal keratin (KRT2E); cytokeratin 2E (CK2E)
U56390; U60521	2.83	caspase 9 (CASP9); MCH6; ICE-like apoptotic protease 6 (ICE-LAP6); apoptotic proteases activating factor 3 (APAF3)
M35543+M57298	2.85	CDC42 homolog; G25K GTP-binding protein (brain isoform+ placental isoform)
U04045; L47583	2.19	DNA mismatch repair protein MSH-2
J03250	4.15	DNA topoisomerase l (TOP1)
X52773	2.03	Retinoid X receptor alpha (RXRA)
X03168	2.20	Vitronectin (VTN); serum spreading factor; S-protein
X94991; X95735	2.78	Zyxin 2(ZYX)
D30751; M22490	2.06	Bone morphogenetic protein 4 (BMP4); BMP2B
D13365; M93311	2.46	Metallothionein lll (MT3); brain growth inhibitory factor (GIFB)
X52541; M62829	2.40	Early growth response protein 1 (EGR1); transcription factor ETR 103; KROX24; zinc finger protein 225(ZNF 225); AT225
X04602; M14584	2.57	Interleukin 6 (IL6); B-cell stimulatory factor 2 (BSF2); interferon beta 2 (IFNB2); hybridoma growth factor (HGF)

Table 9.4.2 Identification by cDNA array of genes negatively regulated by the human recombinant leptin in MCF-7 cells.

Genes were considered negatively regulated when they exhibited a 2-fold or greater decrease in the presence of leptin compared to the absence of leptin treatment. Fold regulation is calculated as the ratio of the level of gene expression in treated with leptin MCF-7 cells to that without leptin treated MCF-7 cells.

Genebank Accession	Fold stimulation	Gene/Protein Name
L27211	0.37	Cyclin-dependent kinase 4 inhibitor 2 (CDK41; CDKN2); P16-INK 4; multiple tumor suppressor 1 (MTS1)
X80692	0.50	Mitogen-activated protein kinase 6 (MAP kinase 6; MAPK 6; PRKM6); p97-MAPK; extracelluar signal-regulated kinase3 (ERK3)
U18671; M97934	0.46	113-kDa signal transducer and activator of transcription 2 (STAT2; STAT113)
L07541	0.50	Replication factor C38-Kda subunit (RFC38); activator 1 38-Kda subunit
M96684	0.37	Purine-rich element-binding protein A (PURA); purine-rich single-stranded DNA-binding protein alpha (PUR-alpha)
X91940	0.35	Wingless-related MMTV integration site 8b protein (WNT8B)
M33294	0.22	Tumor necrosis factor receptor superfamily member 1A (TNFRSF1A); tumor necrosis factor receptor 1(TNFR1); tumor necrosis factor alpha receptor (TNFAR); CD 120A antigen
U72661	0.45	Ninjurin1
U02687	0.30	Stem cell tyrosine kinase 1 (STK1); FL cytokine receptor; tyrosine-protein kinase receptor flt3; CD 135 antigen
X51602; U01134	0.44	Vascular endothelial growth factor receptor 1 (VEGFR1); fms-related tyrosine kinase 1(FLT1); soluble VEGFR; soluble FLT (SFLT)
X57766	0.50	Matrix metalloproteinase 11 (MMP11); stromelysin 3
L20471	0.50	basigin (BSG); leukocyte activation antigen M6; collagenase stimulatory factor; extracellular matrix metalloproteinase inducer (EMMPRIN); 5F7; CD147 antigen
M83246; X51675	0.50	Urokinase-type plasminogen activator receptor GPI-anchored form (U-PAR; PLAUR); monocyte activation antigen MO3; CD87 antigen
D13886; D14705; L23805; L22080	0.32	Alpha 1 catenin (CTNNA1); cadherin-associated protein; alpha E-catenin
U36223	0.42	Fibroblast growth factor 8 (FGF8); androgen-induced growth factor (AIGF); HBGF8
M57627	0.18	Interleukin 10(IL10); cytokine synthesis inhibitory factor (CSIF)
A03911	0.29	Glia-derived neurite-promoting factor (GDNPG)

Chapter 10 Discussion and Conclusion

The present preliminary study demonstrates that human leptin receptor mRNA and protein was expressed in the human breast cancer cell line MCF-7. The results also indicated that the role of leptin on mammary carcinoma cell line proliferation was mediated by the specific leptin receptor in vitro. In addition, cDNA microarray was used to investigate other leptin effects on the MCFR-7 breast cancer cell line.

Previous demonstration of leptin stimulation of the P42/44 MAPK pathway [Baumann, et al, 1996; Bjørbæk, et al, 1997] has been documented. The pathway has been demonstrated to mediate mitogenesis and /or cellular transformation in response to various cellular stimuli. We have demonstrated that Phosphorylation Elk-1 (the p44/42 MAP kinase dependent transcription factor) mediated transcription was enhanced by recombinant leptin stimulation. The present experiments also demonstrated that the addition of human recombinant leptin stimulated MAP kinase actively in dose and time dependent manner, accompanied by an increase in the proliferation of MCF-7 cells. The results obtained for BrdU incorporation showed that DNA synthesis was altered in MCF-7 cells stimulated by leptin. The BrdU assay shows the number of living cells and therefore the results obtained for BrdU uptake may more directly reflect cell proliferation activity and this may explain the difference in the dose-related effect of leptin on MCF-7 cell proliferation.

The present study also demonstrated that leptin stimulated cell proliferation could be completely inhibited with the inhibitor for MEK1/2 (U0126). Particularly, the fact that the addition of U0126 before treatment with leptin also prevented the human recombinant leptin induced increase in cell proliferation indicating the involvement of MAP kinase in MCF-7 cell proliferation. Leptin has been reported to utilize Elk-1 to mediate leptin-induced transcription of egr-1 [Brann et al, 2002], which may provide a possible mechanism for the p44/42 MAP kinase dependent component of leptin-stimulated proliferation.

In cDNA microarray analysis, we have observed the specific increases and decreases in the levels of mRNA expression of MCF-7 cells by leptin stimulation. We have demonstrated that not only Mitogen-activated protein kinases were up regulated in response to leptin, but also Type II cytoskeletal 2 epidermal keratin (KRT2E), Interleukin 6 (IL6); and many cell division genes were up regulated also. While Cyclin-dependent kinase 4 inhibitor 2 (CDK41; CDKN2) Interleukin 10(IL10); cytokine synthesis inhibitory factor (CSIF); Glia-derived neurite-promoting factor (GDNPG) were down regulated, which is consistent with some previous reports [Tartaglia, et al, 1995; Baumann, et al, 1996; White, et al, 1997].

Indeed, leptin has been reported to activate the proliferation of pancreatic-ß cells [Islam,et al, 1997], vascular endothelial cells [Sierra-honigmann, et al, 1998], lung cells [Tsuchiya, et al,1999], gastric mucosa cells [Schneider, et al, 2001], keratinocytes [Stallmeyer, et al, 2001], and preadipose cells [Machinal-Quelin, et al, 2002]. Moreover, higher serum leptin levels are present in obese women who have a higher risk of breast cancer compared to

normal weight women. Thus besides other factors, leptin could be a possible additional factor contributing to mammary epithelium hyperplasia.

A lot of evidence has suggested that the local cellular environment and particularly the mammary adipose cells play an integral role in controlling the proliferation of both normal and neoplastic mammary epithelial cells. Mammary adipose tissue is an important source of paracrine mitogens or anti-mitogens including, insulin-like growth factor 1 (IGF1), transforming growth factor a (TGFa), estrogens, and cytokines (TNFa, IL6). Leptin is another cytokine, mainly produced by adipose tissue and thus may also be relevant.

The preliminary studies of leptin in the mammary carcinoma cell MCF-7 in vitro presented here permit the following conclusions:

The human leptin receptor (OB-Rb) exists in the human breast cancer cell line MCF-7. Human recombinant leptin stimulated MCF-7 cell proliferation by increasing the activity of cell proliferation-related enzymes such as MAP kinase. From the cDNA microarray, it is possible that a number of the regulated genes may be of importance in mediating the effects of leptin proliferation on mammary carcinoma.

This study also suggests that by acting as a proliferation factor on epithelial cell growth, leptin could play directly or indirectly an important role in normal and neoplastic mammary gland growth.

Further work will be necessary to delineate how these genes integrate the response of the cell to leptin. What also needs to be determined is the mechanism and sequential order by which these genes are regulated by leptin.

References 1

1. Adlercreutz H: Western diet and Western diseases: some hormonal and biochemical mechanisms and associations. Scand J Clin Lab Invest 2014: 3-23, 1990.
2. Benichou J, Gail MH. Variance calculations and confidence intervals for estimates of the attributable risk based on logistic models. Biometrics. 1990 Dec; 46(4):991-1003.
3. Bernstein L, Henderson BE, Hanisch R et al (1994) Physical exercise activity reduces the risk of breast cancer in young women. J Natl Cancer Inst 86:1403-1408.
4. Boyle P, Maissoneuve P, Hsieh C. Epidemiology of breast cancer. In: Zatonski W Boyle P, Tyczynski J, editors. Cancer prevention: vital statistics to prevention: proceedings of the International Conference 12-14 October 1988, Warsaw, Pland Warasaw: PA Interpress, 1990:128-35.
5. Bradlow HL, Davis DL, Lin G, Sepkovic D, Tiwari RN Effects of pesticides on the ratio of 16apha/2-hydroxyestrone: a biologic marker of breast cancer risk. Environ Health Perspect 103 (Suppl 7): 147-50, 1995.
6. Brind J, Chinchilli M, et al. Induced abortion as an independent risk factor for breast cancer: a comprehensive review and meta-analysis. J. Epi. and Comm. Health. 10; 50:481-496, 1996.
7. Brinton LA, Benichou J, Gammon MD, Brogan DR, Coates R, Schoenberg JB. Ethnicity and variation in breast cancer incidence. Int J Cancer; 73:349-55. 1997.
8. Bruzzi P, Green SB, Byar DP, Brinton LA, Schairer C: Estimating the population attributable risk for multiple risk factors using case-control data. Am J Epidemiol 122: 904-14, 1985.
9. Buell P: Changing incidence of breast cancer in Japanese-American women. J antl Cancer Inst 51:1479-83, 1973.
10. C. K. Gajalakshmi and V. Shanta. Risk Factors for female breast cancer. Acta Oncological vol. 30 . No 5 1991 569-574
11. Chia K.S., Seow A, Lee H.P. & Shanmugaratnam k., Cancer Incidence In Singapore 1993-1997 (Singapore Cancer Registry Report No.5) 2000
12. Chia K.S., Seow A, Lee H.P. & Shanmugaratnam k., Trends in Cancer incidence in Singapore 1968-1992 (Singapore Cancer Registry Report No.4) 1996
13. Chu SY, Lee NC, Wingo PA, et al. The relationship between body mass and breast cancer among women enrolled in the Cancer and Steroid Hormone Study. J Clin Epidemiol 1991; 44: 1197-1206.
14. Colditz GA, Hankinson SE, Hunter DJ, Willettt WC, Manson JE, Stampfer MJ, Hennekens C, et al. The use of estrognes and progestins and the risk of brast cancer in postmenopausal women. N Engl J Med 1995;332: 1589-93.
15. Daling J, Malone K, et al. Risk of breast cancer among young women: relationship to induced abortion. *JNCI.* 1994; 86:1584-1592.

16. Davidson T Abortion and breast cancer: a hard decision made harder. Lancet Oncol 2001 Dec;2(12):756-8.

17. Dewaard F, Baanders-van Halewijin E (1974) A prospective study in general practice on breast cancer risk in postmenopausal women. Int J Cancer 14:153-160.

18. Dewaard F, Baanders-Van Halewijin EA, Huizinga J. The bimodal age distribution of patients with Mammary carcinoma. Cancer 1964;17:141-52.

19. Dickinson LE, MacMahon B, Cole P, Brown JB: Estrogen profiles of Oriental and Caucasian women in Hawaii. N Engl J Med 291: 1211-3, 1974.

20. Dorgan JF, Brown C, Barrett M et al (1994) Physical activity and risk of breast cancer in the Framingham heart study. Am J Epidemiol 139:662-669.

21. Eugenia E. C, Carmen R, Kimberly W-T, Michael J. T. Overweight, Obesity, and Motality from Cancer in a Prospectively Studied Cohort of U.S. Adults. The New England Journal of Medicine Vol 348: 1625-1638, 2003.

22. Frank D. Gilliland, William C. Hunt, Kathy B. Baumgartmer, et al. Reproductive Risk Factors for Breast cancer in Hispanic and Non-Hispanic White Women. Am J Epidemio. 1998; 148:683-92.

23. Freudenheim JL, Marshall JR, Vena JE, et al: Premenopausal breast cancer risk and intake of vegetable, fruits, and related nutrients. J Natl Cancer Inst 88:340-8,1996.

24. Fries H, Nillius SJ, Pettersson F.Epidemiology of secondary amenorrhea. II. A retrospective evaluation of etiology with special regard to psychogenic factors and weight loss.Am J Obstet Gynecol. 1974 Feb 15;118(4):473-9.

25. Frisch R, Gotz-Welbergen A, McArthur J et al (1981) Delayed menarche and amenorrhea of college athletes in relation to age of onset of training. JAMA 246:1559-1563.

26. Galanis DJ, Kolonel LN, Lee J, Lemarchand L. Anthropometric predictors of breast cancer incidence and survival in a multi-ethnic cohort of female residents of Hawaii, United States. Cancer Causes Control 1998;9:217-24.

27. Glodin BR, Adlercreutz H, Gorbach SL, et al: The relationship between estrogen levels and diets of Caucasian American and Oriental immigrant women. Am J Clin Nutr 44: 945-53, 1986.

28. Goodman MJ, Bulbrook RD, Moore JW: The distribution of estradiol in the sera of normal Caucasian, Chinese, Filipino. Hawaiian and Japanese women living in Hawaii. Eur J Cancer Clin Oncol 24: 1855-60, 1988.

29. Goodman MT, Nomura AMY, Wilkens LR, Hankin JH: The association of diet, obesity, and breast cancer in Hawaii. Cancer Epideiol, Biomarkers Prev 1:269-75,1992.

30. Haenszel W, Kurihara M: Studies of Japanese migrants. I. Mortality from cancer and other diseases among Japanese in the United States. J Natl Cancer Inst 50: 21-42, 1973.

31. Hartmann A, Blaszyk H, Saitoh S, et al: High frequency of p53 gene mutations in primary breast cancers in Japanese women, a low incidence population. Br J Cancer 73: 896-901. 1996

32. Henderson B, Ross R, Bernstern L (1988) Estrogens as a cause of human cancer: The Richard and Hinda Rosenthal Foundation Award Lecture. Cancer Res 48:246-253.

33. Henderson BE, Pike MC, Bernstein L, Ross RK: Breast cancer. In;Schottenfeld D, Fraumeni JFJ, eds. Cancer epidemiology and prevention. 2nd ed. New York: Oxford University Press, 1996: 1022-39.

34. Henderson BE, Ross RK, Rike MC, Casagrance JT: Endogenous hormones as a major factor in human cancer. Cancer Res 42: 3232-9, 1982.

35. Hennekens CH, Julie E. Buring, Epidemiology in Medicine, 1996

36. Hirose K, Tajima K, Hamajima N, et al: effect of body size on breast cancer risk among Japanese women. Int J Cancer 80: 349-55,1999.

37. Ho GH, Luo XW, Ji CY, Foo SC, Ng EH: Urinary 2/16 alpha-hydroxyestrone ratio: correlation with serum insulin-like growth factor binding protein-3 and a potential biomarker of breast cancer risk. Ann Acad Med Singapore 27: 294-9, 1998.

38. Hu Y.H, Nagata C, shimizu H, Kaneda N, Kashiki Y: Association of body mass index, physical activity, and reproductive histories with breast cancer: a case-control study in Gifu, Japan. Breast cancer Res Treat 43: 65-72, 1997.

39. Hunter DJ, Willett WC. Diet, body size, and breast cancer. Epidemiology Rev 15: 110-32, 1993

40. Inoue R, Fukutomi T, Ushijima T, Matsumoto Y, Sugimura T, Nagao M. Germ line mutation of BRCA1 in Japanese breast cancer families. Cancer Res 55:3521-3524 (1995)

41. Janerich DT, Hoff MB, Evidence for a crossover in brat cancer risk factors. Am J Epidemiol 1982; 116:737-42.

42. Kabat GC, Chang CJ. Sparano JA, et al: Urinary estrogen metabolites and breast cancer: a case-control study. Cancer Epidemiol, Biomarkers Prev 6: 505-9, 1997.

43. Kelsey, JL, Fischer DB, Holford TR, et al. Exogenous estrogens and other factors in the epidemiogy of breast cancer. J Natl Cancer Inst 1983; 67:327-33.

44. Key TJA, Chen J, Wang DY, Pike MC, Boreham J: Sex hormones in women in rural China and Britain, Br J Cancer 62:631-6, 1990.

45. Kolonel LN, Nomura AM, Lee J, Hirohata T: Anthropometric indicators of breast cancer risk in postmenopausal women in Hawaii. Nutr Cancer 8: 247-56, 1986.

46. Kvale G, Heuch I (1988). Lactation and cancer risk: is there a relation specific to breast cancer? J Epidemiol Community Health 42: 30-37.

47. Lau K.E. Singapore Census of Population 1990. Statistical Release 1: Demographic Characteristics. Department of Statistics, Singapore 1992.

48. Layde PM, Webster LA, Baughman AL (1989). The independent associations of parity, age at first full-term pregnancy, and duration of breast feeding with the risk of breast cancer. J Clin Epidemiol 42:963-973.

49. Le Marchand L, Kolonel LN, Earle ME, Mi MP. Body size at different periods of life and breast cancer risk. Am J Epidemiology 128: 137-52, 1988.

50. Le Marchand L, Kolonel LN: Cancer in Japanese migrants to Hawaii: interaction between genes and environment]. Rev Epidemiology Sante Publique 40: 425-30, 1992.

51. Le Marchand L, sivaraman L, Pierce L, et al: Association of CYP1A1, GSTM1, land CYP2E1 polymorphisms with lung cancer suggest cell type specificties to tobacco carcinogens. Cancer Res 58: 4858-63, 1998.

52. Lee H.P, Lynn G, Stephen W. D, Jacques E, James L, Nicholoas, E.D. Risk factors for breast cancer by age and menopausal status: a case-control study in Singapore. Cancer Causes and Control. 3, Vol. 1992. 313-322.

53. Lee H.P, Lynn G, Stephen W. D,, Jacques E, James L, Nicholoas, E.D Dietary effects on breast cancer risk in Singapore. Epidemiology Vol 337: May 18, 1991, 1197-1200.

54. Lubin JH, Burns PE, Blot WJ, Lees AW, May C, Morris LE, et al. Risk factors for breast cancer in women in northern Alberta, Canada, as related to age at diagnosis. J Natl Cancer Inst 1982; 68:211-7.

55. Maclure M, Travis LB, Willettt W, et al. A prospective cohort study of nutrient intake and age at menarche. Am J Clin Nutr;54:649-56, 1991.

56. Madigan MP, Ziegler RG, Benichou J, Byrne C, Hoover RN: Proportion of breast cancer cases in the United States explaining by well-established risk factors. J Natl Cancer Inst 87: 1681-5,1995.

57. Mayberry RM, Age-specific patterns of association between breast cancer and risk factors in black women, ages 20 to 39 and 40 to 54. Ann Epidemiol 1994; 4:205-13.

58. Messina M, Barnes S: The role of soy products in reducing risk of cancer. J Natl Cancer Inst 83: 541-6, 1991.

59. Michnovicz JJ, Bradlow HL: Altered estrogen metabolism and excretion in humans following consumption of indole-3-carbinol. Nutr Cancer 16: 59-66, 1991.

60. Miller BA, Feuer EJ, Hankey BF. The increasing incidence of breast cancer since 1982; relevance of early detection. Cancer Causes Control 1991;2:67-74.

61. Miller BA, Gloeckler Ries LA, Hankey BF, Kosay CL, Harras A, Devesa SS, et al. Annual cancer statistics review. SEER Program, Washington, DC: National Cancer Advisory Board; 1993. NIH publication No. 93-2789.

62. Nebert DW: Elevated estrogen 16a–hydroxylase activity: is this a genotoxic or nongenotoxic biomarker in human breast cancer risk? J Natl Cancer Inst 85: 1888-91, 1993.

63. Newcomb PA, Storer BE, Longencker MP et al (1994) Lactation and a reduced risk of premenopausal breast cancer. N Engl J Med 330:81-87.

64. Newcomb RA, Storer BE, Longnecker MP, Mittendorf R, Greenberg ER, Willettt WC (1996) Pregnancy termination in relation to risk of breast cancer. JAMA 275:283-287.

65. Ng EH, Gao F, Ji CY, Ho GY, Soo KC. Risk factors for breast carcinoma in Singaporean women. Cancer 1997;80:725-31.

66. Nomura AM. Kolonel LN, Hirohata T, Lee J: the association of replacement estrogens with breast cancer. Int J Cancer 37; 49-53. 1986.

67. Nomura AMY, Henderson BE, Lee J. Breast cancer and diet among Japanese in Hawaii. Am J Clin Nutr 31:2020-5,1978.

68. Nomura AMY. Lee J. Kolonel LN. Hirohata T: Breast cancer in two populations with different levels for the disease. Am J Epidemiol 119:496-502, 1984.

69. Obermeier MT, White RE, Yang CS: Effects of bioflavonoids on hepatic P450 activities. Xenobiotica 25:575-84, 1995.

70. Palmer JR, Rosenberg L, Rao RS, Strom BL, Warshauer ME, Harlap S, et al. Oral contraceptive use and breast cancer risk among African-American women. Cancer Causes Control 1995; 6:321-31.

71. Parkin D M, Muir C S, Whelan S L, Gao Y T, Ferlay J, Powell J. Cancer Incidence in Five Contents. Vol VI. IARC Scientific Publication No. 120. IARC, Lyon, 1992.

72. Pathak DR, Speizer FE, Willett WC, Rosner B, Lipnick RJ. Rarity and breast cancer risk: possible effect on age at diagnosis. Int J Cancer 1986;37:21-5.

73. Pathak DR, Whittemore AS. Cobined effects of body size, parity, and menstrual events on breast cancer incidence in seven countries. Am J Epidemiol 1992; 135: 153-68.

74. Perera FP. Molecular epidemiology in cancer prevention. In: Schottenfeld D, Fraumeni JF, eds. Cancer Epidemiology and Prevention. 2nd edition. New York, Oxford: Oxford University Press, 1996: 101-15.

75. Pike MC, Krailo MD, Henderson BE, Casagrande JT, Hoel DG: 'Hormonal' risk factors, 'breast tissue age' and the age-incidence of breast cancer. Nature 303:767-70, 1983.

76. Pike MC. Spicer DV, Dahmoush L, Press MF: Estogens. Progestogens, normal brast cell proliferation, and breast cancer risk. Epidemiol Rev 15; 17-35m 1993.

77. Prentice RL, Sheppard L. Dietary fat and cancer: consistency of the epidemiologic data and disease prevention that may follow from a practical reduction in fat consumption. Cancer Causes Control 1990; 1:81-97.

78. Preston Martin s, Pike MC, Ross RK, Jones PA, Henderson EB, et al. Increased cell division as a cause of human cancer. Cancer Res 1990; 50: 7415-21.

79. Ries LAG, Miller BA, Hankey BF et al (eds) (1994) SEER Cancer Statistics Review, 1973-1991. (NIH publication 94-2789) US DHHS National Cancer Institute, Bethesda.

80. Rockhill B, Weinberg CR, Newman B: Population attributable fraction estimation for established breast cancer risk factors: considering the issues of high prevalence and unmodifiablility. Am J Epidmiol 147: 826-33, 1998.

81. Rosner B, Colditz GA, Willett WC. Reproductive risk factors in a prospective study of breast cancer: the Nurses' Health Study. Am J Epidemiol 1994; 139: 819-35.

82. Russo J, Russo IH. Hormonal induced differentiation: a novel approach to breast cancer prevention. J Cell Biochem Suppl 1995;22:58-64.

83. Russo J, Russo IH. Toward a physiological approach to breast cancer prevention. Cancer Epidemiology, Biomarkers and Prevention. 1994; 3:353-364.

84. Sanderson M, Shu XO, Jin F, Dai Q, Wen W, Hua Y, Gao YT, Zheng W. Abortion history and breast cancer risk: results from the Shanghai Breast Cancer Study. Int J Cancer 2001 Jun 15;92(6):899-905

85. Seidman H, Stellman SD, Mushinski MH: A different perspective on breast cancer risk factors: some implications of the nonattributable risk. CA Cancer J Clin 32: 301-13, 1982.

86. Seow A, Duffy S W, Mcgee, M A, Lee J and Lee H.P. Breast cancer in Singapore: Trends in incidence 1968-1992. International Journal of Epidemiology 1996 Vol 25, No.1, 40-45.

87. Shapiro TA, Fahey JW, Wade KL, Stephenson KK, Talalay P: Human metabolism and excretion of cancer chemoprotective glucosinolates and isothiocyanates of cruciferous vegetables. Cancer Epidemiol, Biomarkers Prev 7:1091-100, 1998.

88. Shimizu H, Ross RK, Bernstein L, et al. Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. Br j Cancer 1991;63:963-6.

89. Shimizu H. Ross RK, Bernstein L, Pike M. Henderson BE: Serum estrogen levels in postmenopausal women: comparison of American whites and Japanese in Japan. Br J Cancer 62:451-3. 1990.

90. Smith-Warner SA, Spiegelman D, Hunter DJ, for the Pooling Project of Prospective Studies of Diet and Cancer Investigators. Population attributable risk of breast cancer due to reproductive factors (Abstract). Cancer Epidemiol, Biomarkers Prev 7: 176, 1998.

91. Sng JH, Chang J, et al. The prevalence of BRCA1 mutations in Chinese patients with early onset breast cancer and affected relatives. Br J cancer 2000; 82:538-42.

92. Spicer DV, Pike MC, Epidemiology of breast cancer. In: Lobo RA., editor. Treatment of the postmenopausal women: basic and clinical aspects. New York: Raven Press, 1994.

93. Tao SC, Yu MC, Ross RK, Xiu KW. Risk factors for breast cancer in Chinese women of Beijing. Int J Cancer 1988; 42: 495-8.

94. Thomas DB, Karagas MR. Cancer in first and second generation Americans. Cancer Res 1987; 47:5771-6.

95. Topper YJ, Freeman CS. Multiple hormone interactions in the developmental biology of the mammary gland. Physilo Rev 1980;60:1049-106.

96. Trichopoulos D, Lipman RD. Mammary gland mass and breast cancer risk. Epidemiology 3: 523-6, 1992.

97. Trichopoulos D, Yen S, Brown J, Cole P, Macmahon B: The effect of westernization on urine estrogens, frequency of ovulation, and breast cancer risk. A study of ethnic Chinese women in the Orient and the USA. Cancer 53:187-92, 1984.

98. Ursin G. London S, Stanczyk FZ, et al: Urinary 2-hydroxyestrone/ 16alpha-hydroxyestrone ratio and risk of brast cancer in postmenopausal women. J Natl Cancer Inst 91: 1067-72, 1999.

99. Waterhouse J, Muir C, Shanmugaratnam K, Powell J. Cancer Incidence in Five Continents. Vol .IARc Scientific Publication 15, IARC, Lyon, 1993.

100. Wu AH, Stanczyk FZ, et al. Soy intake and other lifestyle determinants of serum estrogen levels among postmenopausal Chinese women in Singapore. Cancer Epidemiol Biomarkers Prev. 2002; 11:844-51.

101. Wu AH, Ziegler RG, Horn-Ross PL, et al: Tofu and risk of breast cancer in Asian-Americans. Cancer Epidemiol Biomarkers Prev 5:901-6, 1996.

102. Wynder EL, Fujita Y, Harris RE, Hirayama T, Hiyama T: Comparative epidemiology of cancer between the United States and Japan. A second look. Cancer 67:746-63, 1991.

103. Yager JD, Iiehr JG: Molecualr mechanisms of estrogen carcinogenesis. Annu Rev Pharmacol Toxicol 36: 203-32, 1996.

104. Yip CH, Ng EH. Breast cancer--a comparative study between Malaysian and Singaporean women. Singapore Med J. 1996 Jun;37(3):264-7.

105. Yuan JM, Wang Q-S, Ross RK, Henderson BE, Yu MC: Diet and breast cancer in Shanghai and Tianjin, China. Br J Cancer 71:1353-8,1995.

106. Zidgler RG, Hoover RN, Nomura AMY, West DW, WuAH, Pike MC, et al. Relative weight, weight change, height, and breast cancer risk in Asian-American women. J Natl Cancer Inst 1996; 88:650-60.

References 2

1. Banks A.S., Davis S.M., Bates S.H. and Myers M.G., Activation of downstream signals by the long form of the leptin receptor. J Biol Chem 275 (2000), pp. 14563-14572.

2. Baumann H., Morella K.K., White D.W., Dembski M., Bailon P.S., Kim H., Lai C.F. and Tartaglia L.A., The full length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors. *Proc Natl Acad Sci USA* 93 (1996), pp. 8374–8378.

3. Bjø rbæk C., Elmquist J.K., Frantz J.D., Shoelson S.E. and Flier J.S., Identification of SOCS-3 as a potential mediator of central leptin resistance. Mol Cell 1 (1998), pp. 619-625.

4. Bjø rbæk C., Haschimi K. E, Frantz J.D. and Flier J.S., The role of SOCS-3 in leptin signaling and leptin resistance. J. Biol Chem 274 (1999), pp. 30059-30065.

5. Bjø rbæk C., Lavery H.J., Bates S.H., Olson R.K., Davis S.M., Flier J.S. and Myers M.G., SOCS3 mediates feedback inhibition of the leptin receptor via Tyr985. J Biol Chem 275 (2000), pp. 40649-40657.

6. Bjø rbæk C., Uotani S., Silva B. da and Flier J.S., Divergent signaling capacities of the long and short isoforms of the leptin receptor. J Biol Chem 272 (1997), pp. 32686-32695.

7. Brann. DW, Wade, MF, Dhandapani, KM, Mahesh, VB, Buchanan, CD. Leptin and reproduction. Steroids 67 (2002) 95-104.

8. Butte N.F. , Hopkinson J.M. and Nicolson M.A., Leptin in human reproduction: serum leptin levels in pregnant and lactating women. J Clin Endocrinol Metab 82 (1997), pp. 585-589.

9. Campfield L.A., Smith F.J., Y., Guisez R. Devos and Burn P., Recombinant mouse ob protein: evidence for a peripheral signal linking adiposity and central neural networks. Science 269 (1995), pp. 546-549.

10. Carpenter L.R., Farruggella T.J., Symes A., Karow M.L., Yancopoulos G.D. and Stahl N., Enhancing leptin response by preventing SH2-containing phosphatase 2 interaction with OB receptor. Proc Natl Acad Sci USA 95 (1998), pp. 6061-6066.

11. Chehab F, Mounzih K, Lim ME. Early onset of reproductive function in normal mice treatd with leptin. Science 1996; 275:88-90.

12. Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Mardco CC, McKee LJ, Bauer TL, Caro JF. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 1996, 334, 292-295.

13. Cusin, A. Sainsbury, P. Doyle, F. Rohner-Jeanrenaud and B. Jeanrenaud, The ob gene and insulin - a relationship leading to clues to the understanding of obesity. Diabetes 44 (1995), pp. 1467-1470.

14. Frederich Rc, Hamann A, Anderson S, Lollman B, Lowell B, Fleir J. Leptin levels reflect levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. Nature Med 1995, 1, 1311-1314.

15. Frederich RC, Lollman B, Hamann A, Napolitino-Rosen A, Kahn BB, Lowell BB, Flier JS. Expression of ob mRNA and its encoded protein in rodents: impact of nutrition and obesity. J Clin Invest 1995, 96, 1658-1663.

16. Ghilardi N. and Skoda R.C., The leptin receptor activates janus kinase 2 and signals for proliferation in a factor-dependent cell line. Mol Endocrinol 11 (1997), pp. 393-399.

17. Ghilardi N., Ziegler S., Wiestner A., Stoffel R., Heim M.H. and Skoda R.C., Defective STAT signaling by the leptin receptor in diabetic mice. Proc Natl Acad Sci USA 93 (1996), pp. 6231-6235.

18. Gluckman PD, Heyman MA, editors, Scientific basis of pediatric and perinatal medicine, 2nd Edition, London: Edward Arnold Publisher, 1996.

19. Grunfeld C, Zha C, Fuller J, Pollack A, Moser A, Friedman J, Feingold KR, Endotoxin and cytokines induce expression of leptin, the ob gene product in hamsters. J Clin Invest 1996, 97, 2152-2157.

20. Halaas J.L, Gajiwala K.S., Maffei M. et al., Weight-reducing effects of the plasma protein encoded by the obese gene. Science 269 (1995), pp. 543-546.

21. Hamann and S. Matthei, Regulation of energy balance by leptin. Exp Clin Endocrinol Diabetes 104 (1996), pp. 293-300.

22. Hamilton BS, Paglia D, Kwan ATM, Deitel M, Increased obese mRNA expression in omental fat cells from massively obese humans. Nature Med 1995, 1, 953-956.

23. Islam M.S., Morton N.M., Hansson A. and Emilsson V., Rat insulinoma-derived pancreatic beta-cells express a functional leptin receptor that mediates a proliferative response. Biochem. Biophys. Res. Commun. 238 (1997), pp. 851-855.

24. Kaulsay Karmal K., Hicheem C. Mertani, Kok-Onn Lee, and Peter E. Lobie. Autocrine human growth hormone enhancement of human mammary carcinoma cell spreading is Jak2 Dependent. Endocrinology Vol 141 No.4 1571-1584.

25. Kopp W, Blum WF, Prittwitz S, Ziegler A, Lubbert H, Emons G et al. Low leptin levels predict amenorrhea in underweight and eating disordered females. Molec Psychiatry 1997;2:335-40.

26. Lahlou N, Landais P, De-Boissieu D, Bougneres PF. Circulating leptin in normal children and during the dynamic phase of juvenile obesity: relation to body fatness, energy metabolism, caloric intake, and sexual dimorphism. Diabetes 1997; 46:989-93.

27. Li C. and Friedman J.M., Leptin receptor activation of SH2 domain containing protein tyrosine phosphatase 2 modulates Ob receptor signal transduction. Proc Natl Acad Sci USA 96 (1999), pp. 9677-9682.

28. Lonnqvist F, Arner P, Nordfors L, Shalling M. Over expression of the obese (ob) gene in adipose tissure of human obese subjects. Nature Med 1995, 1, 950-953.

29. Machinal-Quelin F., Dieudonne M.N., Leneveu M.C., Pecquery R., Giudicelli Y., In vitro proadipogenic effect of leptin on rat preadipocytes: activation of mitogen-activated protein kinase and of STAT3 signaling pathways, Am. J. Physiol. 2002.

30. Madej T, Boguski MS, Bryant SH, Threading analysis suggests that the obese gene product may be a helical cytokine. FEBS Lett 373, 13-18.

31. Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, Kern PA, Friedman JM. Leptin levels in human and rodent: Measuremnt of plasm leptin and ob RNA in obese and weight reduced subjects. Nature Med 1995, 1, 1151-1161.

32. Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, Kern PA, Friedman JM. Incrased expression in adipocytes of ob RNA in mice with lesions of the hypothalamus and with mutations at the db locus. Proc Natl Acad Sci USA 1995, 92, 6957-6960.

33. Murakami M., Narazaki M., Hibi M., Yawata H., Yasukawa K., Hamaguchi M., Taga T. and Kishimoto T., Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family. *Proc Natl Acad Sci USA* 88 (1991), pp. 11349–11353.

34. Murakami T., Yamashita T., Iida M., Kuwajima M. and Shima K., A short form of leptin receptor performs signal transduction. Biochem Biophys Res Commun 231 (1997), pp. 26-29.

35. Pelleymounter M.A., M.J. Cullen, M.B. Baker, R. Hecht, D. Winters, T. Boone and F. Collins, Effects of the obese gene product on body weight in ob/ob mice. *Science* 269 (1995), pp. 540–543.

36. Rosenblum C.I., Tota M., Cully D., Smith T., Collum R., Qureshi S., Hess J.F., Phillips M.S., Hey P.J., Vongs A., Fong T.M., Xu L., Chen H.Y., Smith R.G., Schindler C. and Van L.H. der Ploeg , Functional STAT1 and 3 signaling by the leptin receptor (OB-R); reduced expression of the fatty leptin receptor in transfected cells. Endocrinology 137 (1996), pp. 5178-5181.

37. Saladin R, De Vos P, Guerre-Millo M, Leturque A, Girard J, Staels B, Auwerx J. Transient increase in obese gene expression after food intake or insulin administration. Nature 1996, 377, 527-529.

38. Schneider R., Bornstein S.R., Chrousos G.P., Boxberger S., Ehninger G. and Breidert M., Leptin mediates a proliferative response in human gastric mucosa cells with functional receptor. Hormone Metab. Res. 33 (2001), pp. 1-6.

39. Sierra-honigmann M.R., Nath A.K., Murakami C., Garciacardena G., Papapetropoulos A., Sessa W.C., Madge L.A., Schechner J.S., Schwabb M.B., Polverini P.J. and Floresriveros J.R., Biological action of leptin as an angiogenic factor. Science 281 (1998), pp. 1683-1686.

40. Sinha M.K., Human leptin: the hormone of adipose tissue. Eur J Endocrinol 136 (1997), pp. 461-464.

41. Slieker LJ, Sloop KW, Surface PL, Kriauciunas A, La QuierF, Manetta J, Bue-Valleskey J, Stephens TW. Regulation of expression of ob mRNA and protein by a glucocorticoids and camp. J Biol Chem 1996, 271, 5301-5304.

42. Spitzweg C. and A.E. Heufelder, More clues from fat mice: leptin acts as an opponent of the hypothalamic neuropeptide Y system. Eur J Endocrinol 136 (1997), pp. 590-591.

43. Stallmeyer B., Kampfer H., Podda M., Kaufmann R., Pfeilschifter J. and Frank S., A novel keratinocyte mitogen: Regulation of leptin and its functional receptor in skin repair. J. Invest. Dermatol. 117 (2001), pp. 98-105.

44. Starr R., Willson T.A., Viney E.M., Murray L.J., Rayner J.R., Jenkins B.J., Gonda T.J., Alexander W.S., Metcalf D., Nicola N.A. and Hilton D.J., A family of cytokine-inducible inhibitors of signalling. Nature 387 (1997), pp. 917-921.

45. Stephens T.W, M. Basinski, P.K. Bristow, J.M. Bue-Valleskey, S.G. Burgett, L. Craft et al., The role of NPY in the antiobesity action of the obese gene product. Nature 377 (1995), pp. 530-532.

46. Takahashi et al., 1997. Y. Takahashi, Y. Okimura, I. Mizuno, K. Iida, T. Takahashi, H. Kaji, H. Abe and K. Chihara , Leptin induces mitogen-activated protein kinase-dependent proliferation of C3H10T1/2 cells. J. Biol. Chem. 272 (1997), pp. 12897-12900.

47. Tanabe et al., 1997. K. Tanabe, S. Okuya, Y. Tanizawa, A. Matsutani and Y. Oka, Leptin induces proliferation of pancreatic βcell line MIN6 through activation of mitogen-activated protein kinase. Biochem. Biophys. Res. Commun. 241 (1997), pp. 765-768.

48. Tartaglia et al., 1995. L.A. Tartaglia, M. Dembski, X. Weng, N. Deng, J. Culpepper, R. Devos, G.J. Richards, L.A. Campfield, F.T. Clark, J. Deeds et al., Identification and expression doning of a leptin receptor, OB-R. Cell 83 (1995), pp. 1263-1271.

49. Tartaglia L.A., The leptin receptor. J Biol Chem 272 (1997), pp. 6093-6096.

50. Tomaszuk, C. Simpson and G. Williams, Neuropeptide Y, the hypothalamus and the regulation of energy homeostasis. Horm Res 46 (1996), pp. 53-58.

51. Tsuchiya T., Shimizu H., Horie T. and Mori M., Expression of leptin receptor in lung: leptin as a growth factor. Eur. J. Pharmacol. 365 (1999), pp. 273-279.

52. Uotani S., Bjø rbæk C., Tornoe J. and Flier J.S., Functional properties of leptin receptor isoforms: internalization and degradation of leptin and ligand-induced receptor down-regulation. Diabetes 48 (1999), pp. 279-286.

53. Vaisse C., Halaas J.L., Horvath C.M., Darnell J.E., Stoffel M. and Friedman J.M., Leptin activation of STAT3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. Nat Genet 14 (1996), pp. 95-97.

54. White D.W., Kuropatwinski K.K., Devos R., Baumann H. and Tartaglia L.A., Leptin receptor (OB-R) signaling. Cytoplasmic domain mutational analysis and evidence for receptor homo-oligomerization. J Biol Chem 272 (1997), pp. 4065-4071.

55. Yamashita T., Murakami T., Otani S., Kuwajima M. and Shima K., Leptin receptor signal transduction: OBRa and OBRb of fa type. Biochem Biophys Res Commun 246 (1998), pp. 752-759.

56. Zhang Y., Proenca R., Maffei M., Barone M., Leopold L. and Friedman J.M., Positional cloning of the mouse obese gene and its human homologue. Nature 372 (1994), pp. 425-432.

Appendix

Questionnaire

(Woman's Health Research)

Date of interview ☐☐/☐☐/☐☐
m m d d y y

Date of diagnosis ☐☐/☐☐/☐☐
m m d d y y

Nationality group ☐
1 Chinese
2 Malay
3 Indian
4 Other_____

If you are not a Singapore resident, which year did you come to Singapore?
☐☐☐☐

Country of birth ☐
1 Singapore
2 Malaysia
3 P R China
4 India
5 Other_____

Date of birth ☐☐/☐☐/☐☐
m m d d y y

	Hormonal Factors	Please fill in your answer
H1	Do you still have the regularity menstrual period? 1=Yes (skip to next section) 2=No	☐
H2	If you have not regularity menstrual period, the last time is	☐☐/☐☐ **m m y y**
H3	How old were you when you had your first regular menstrual period?	☐☐ years old
H4*	How often are/were your menstrual periods? Record cycle length in days (e.g. once in 28 days) If less often than monthly, record approximate number of days (e.g. 60 for 2-monthly).	Once in ☐☐ days
H5	Have you taken any medicine (oral contraceptive/ estrogen pill/ traditional Chinese medicine) that interrupted your menstrual cycling? 1=no (skip to next section) 2=yes	☐
H6	How long did you take it? The total months is	☐☐ months
H7	Your marital status is 1=single (skip to next section) 2=married 3=divorce 4=widowed 5=separated	☐
H8	How old were you when you first married?	☐☐ years old

H9	How many children do you have? 0=null (skip to next section) 1=1 child 2=2 children 3=3 children 4=more than 3 children	☐
H10	When was your First Full Term Pregnancy?	☐☐ years old
H11	If you gave your child/ children breast-feeding, the total months is_____ If you have 2 children, the breast-feeding time is the months of 1st child plus the months of 2nd child.	☐☐ months
H12	Have you ever taken oral contraceptives / female hormone (pills/ implants)? 1=never (skip to next section) 2=ever	☐
H13	If you have taken oral contraceptives/ female hormone (pills/implants), it is/was 1=in regularity menstrual period 2=in the period of menstruation ceasing (have not menstrual at least 6 months) 3=in irregularity menstrual period	☐
H14	If you have taken oral contraceptives/ female hormone (pills/implants), the total years is/was	☐☐ months

*Pregnancy	Year	Outcome 1=Full term baby (>36 weeks) 2=Premature baby 3=miscarriage 4=abortion 5=others, specify_____	If the outcome is not full term baby, when did the termination of pregnancy occur?
1	_____	☐	_____months
2	_____	☐	_____months
3	_____	☐	_____months
4	_____	☐	_____months
5	_____	☐	_____months
6	_____	☐	_____months

	Socioeconomic Status	**Please fill in your answer**
S1	Your highest level of your educational qualifications is 1=null of primary 2=Primary-school 3=Secondary –school 4=Polytechnic 5=Bachelor 6=Master 7=PhD.	☐
S2	Which kind of house do you live in? 1=HDB/ JTC/ Other Government 1~3-room flat (include shophouse, attap/ Zinc-roofed dwelling) 2=HDB 4-room flat 3=HDB 5-room flat 4=HDB executive flat 5=Private apt or condominium 6=Terrace/ semi-detached/ bungalow	☐
S3	Do your family own a car? 1=yes 2=no	☐

	Other factors	
F1	Has anyone in your family ever had cancer? 1=no (skip to next section) 2=yes	☐
F2	Was it your _____? 1=Parent, sibling or child 2=husband or husband's family 3=other relative _____	☐
F3	What type of cancer was it? 1=breast 2=other (specify_____)	☐
F4	Have you had a history of breast lump? 1=no (skip to next section) 2=yes	☐
F5	What kind of disease was it? Specify_____	
F6	When did you have the regular breast examination last time?	☐☐/☐☐ **m m y y**
F7	Is there any abnormal on it? 1=no 2=yes (specify_____)	☐
F8	What is your height currently?	☐☐☐ cm
F9	What was your height at age 18?	☐☐☐ cm
F10	What is your weight currently?	☐☐ kg
F11	What was your weight at age 18?	☐☐ kg

F12	Have you ever smoked a cigarette (or any other form of tobacco at least one a day for a year)? 1=never (skip to next section) 2=ever, hand rolled cigarette 3=ever, manufactured cigarette 4=ever, other form (specify_____)	☐
F13	At what age did you start smoking?	☐☐ years old
F14	At what age did you stop smoking?	☐☐ years old
F15	On average, how many sticks did you smoke in a day?	☐☐ sticks
F16	Have you ever taken alcohol (beer/wine/ brandy/ spirits/ liquor)? 1=never (skip to next section) 2=ever	☐
F17	At what age did you start drinking alcohol?	☐☐ years old
F18	At what age did you stop drinking alcohol?	☐☐ years old
F19	On average, how many glasses/shots did you drink in a week/ month? (25ml/cup)	☐☐ glasses/shots per week/month

Note: * For interview

In case we need to clarify some of this information with you could I have your contact number? ☐☐☐☐☐☐☐

Thank you for your help!

Patient's Consent

I, _____agree to participate in this research project conducted by the National University of Singapore on ***Woman's health research***. I understand that I am asked to provide information about matters concerning myself and my health, and that the doctors involved in this study may require further information from my medical records. I also understand that all this information will be kept confidential and available only to the doctors involved in the study.

Signed:_____ Witnessed by_____

Date:_____ _____

(Name of witness)